Evaluation of refrigerated platelet concentrates supplemented with low doses of second messenger effectors


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Summary

With the goal of producing haemostatically effective platelet concentrates (PCs) with a longer shelf-life, we aimed to identify a simple combination of platelet inhibitors, with a low pharmacological load, which could avoid the unacceptable loss of platelets stored under refrigerated conditions. PCs stored with different combinations of second messenger effectors were analysed at days 5, 10 and 15 of storage and compared with those supplemented with ThromboSol – a combination of six platelet inhibitors that protects cells from cold damage. The following parameters were analysed: platelet counts, biochemical parameters (glucose, pH, bicarbonate, lactate), cell lysis (lactic dehydrogenase, LDH), membrane glycoproteins (GPs), platelet aggregation, fibrinogen binding and hypotonic shock response. We characterized the combination of amiloride and sodium nitroprusside (at 1/2 the dose included in ThromboSol). This was found to be similar to ThromboSol and superior to nontreated units in the prevention of cold-induced platelet aggregation at day 15 of storage (maintenance of 78% and 80% of initial platelet counts, respectively), preservation of GPIbα (11% and 12% better maintenance of mean fluorescence intensity compared with control units, respectively), and reduced cell lysis (13% and 11% decrease in supernatant LDH, respectively). The reduced pharmacological load with the identified solution compared with ThromboSol is an argument in favour of the potential use of these agents when designing strategies to improve PC storage.

Keywords

ThromboSol, cold storage, platelet concentrate, amiloride, sodium nitroprusside

Introduction

Allogeneic platelet concentrates (PCs) are stored in a suspending volume of 45–65 ml, at 22 °C, with an approved storage life being limited to 5 days, because longer storage leads to unacceptable increase in the risk of bacterial contamination (Klinger, 1996; Seghatchian & Krailadsiri, 1997; Pietersz, Engelfriet & Reesink, 2003). The limitation of the storage time of PCs results in severe compromises in platelet inventories and chronic shortages of the product in blood banks. In this regard, routine surveillance of PCs for bacterial contamination has led to extension of the storage time to 7 days in some European centres, while it is expected that the implementation of such tests within the USA will lead to FDA approval for the 7-day prolonged storage time. The 7 day extension of the storage time has resulted in a 60% reduction in the outdating of PCs (Pietersz et al., 2003), leading to obvious advantages on the logistics of blood transfusion services.

A simple and inexpensive approach to prolong the shelf-life of PCs that would obviate the need for bacterial detection is refrigerated storage, since cold inhibits the growth of most strains of bacteria potentially present in PCs (Vostal & Mondoro, 1997). In this regard, the risk of bacterial infections transmitted through refrigerated red
blood cell products is estimated to be 50X lower than that of PC transfusion (McCullough, 2003). There have been few studies involving transfusion of cold-treated platelets in vivo. Transfusion of thrombocytopenic patients (Becker et al., 1973) and aspirin-treated normal subjects (Becker et al., 1973; Valeri, 1974) with platelets stored at refrigerator temperatures for 24–72 h show correction of prolonged bleeding time almost immediately on transfusion, even more rapidly than observed with room temperature-stored platelets. However, storage at 4 °C for 24 h induces a significant reduction in platelet lifespan compared with 24 h room temperature storage (survival of infused platelets of 2 days vs. 8 days, respectively) (Slichter & Harker, 1976). Unfortunately, cold storage affects virtually every aspect of platelet morphology and physiology, collectively referred to as cold storage lesion (Becker et al., 1973; Valeri, 1974). Exposure of platelets to cold temperatures can lead to the induction of spontaneous aggregation after the platelets have been rewarmed, thus leading to experimentally decrease of cell number as a result of aggregate formation (Kattlove & Alexander, 1971), and clustering of the glycoprotein (GP) Ib-receptor complex (Hoffmeister et al., 2003a). Other changes to cold temperatures are analogous to activation of platelets with physiological agonists (Vostal & Mondoro, 1997). These include variation in microtubule arrangements, reduced adenosine diphosphate (ADP)-induced aggregation, a decline in hypotonic shock response (HSR), and increase in β-thromboglobulin release (White & Krivit, 1967; Handin, Fortier & Valeri, 1970; Moroff & Chang, 1979; Snyder et al., 1981; Fagiolo et al., 1989; Murphy & Gardner, 1991). Nowadays, advances in understanding the mechanism of the effects of cold temperature on platelets have led to potential strategies to prevent their occurrence, and have again raised the possibility of an effective liquid cold storage of platelets (Vostal & Mondoro, 1997; Hoffmeister et al., 2003b). One of these strategies has been the supplementation of PCs with ThromboSol (LifeCell Corp., Woodlands, TX, USA), a mixture of selected second messenger effectors that inhibits specific activation pathways in platelets, resulting in cells that in vitro are found to be biochemically stabilized, while apparently protected against bacterial growth and the cold storage damage (Connor et al., 1996; Currie et al., 1997; Rivera et al., 1999).

An unresolved question in this issue is whether or not all components making up ThromboSol are fully required for protection of platelets against deleterious cold effects. Our study aimed to gain further insights into the effectiveness of each ThromboSol reagent for the prevention of the cold-induced platelet damage, and to develop a simpler combination of second messenger effectors that would still provide a protection to platelets from cold damage.

Materials and methods

Material

Monoclonal antibodies (mAbs) to platelet glycoproteins (GPs) GPIIb/IIIa (FITC-CD42) GPIIb/IIa (FITC-CD61), and P-selectin (PE-CD62) were purchased from Becton Dickinson (San Jose, CA, USA), and to granulophysin (FITC-CD63) was from Immunotech (Marseille, France). A modified ThromboSol solution was prepared in dimethyl sulphoxide (DMSO) as a 50X concentration, containing amiloride (12.5 mm), adenosine (5 mm), sodium nitroprusside (SNP; 2.5 mm), dipyridamole (2 mm) and quinacrine (10 μm). Ticlopidine was not included, as it has been shown to have no in vitro antiplatelet effects (Saltiel & Ward, 1985). All other chemicals and solvents were of the highest analytical grade commercially available.

Preparation, storage and sampling of PCs

Whole blood (450 ml) from volunteer blood donors was collected in conventional triple blood collection systems (Laboratorios Grifols SA, Barcelona, Spain), in which the primary pack contained 63 ml of citrate phosphate dextrose (CPD). The platelet container is made of polyvinyl chloride (PVC), and according to the manufacturer, has adequate gas exchange properties.

Platelet concentrates were obtained from whole blood units by stepwise centrifugation by the platelet rich plasma (PRP) method as previously described (Rivera et al., 1994). A preliminary set of six different pools of PCs was carried out to assess the individual capacity of each ThromboSol reagent to prevent the cold-induced loss of platelet counts in refrigerated PCs. In each of these pools, seven ABO isogroup PCs were mixed by the use of a sterile device (SCD; Haemonetics, Braintree, MA, USA), and leukoreduced by filtration (LRP10; Pall Corporation, New York, NY, USA). Then, pooled platelets were distributed into separated containers (400 ml PVC-TOTM, Laboratorios Grifols SA, Barcelona, Spain) of 50 ml volume. One bag (bag 6) was injected with 1 ml of 50X ThromboSol via a sampling-site coupler (Baxter SA, Barcelona, Spain) to achieve a final 1X ThromboSol concentration. Each remaining bag received 1 ml of either 50X individual reagent of ThromboSol, resulting in the following final concentrations: bag 1, 0.25 mm amiloride; bag 2, 0.25 mm amiloride; bag 3, 2.5 mm adenosine; bag 4, 2.5 mm adenosine; bag 5, 2.5 mm adenosine; bag 6, ThromboSol.
50 μM SNP; bag 3, 0.1 mM adenosine; bag 4, 40 μM dipyridamole; bag 5, 0.2 μM quinacrine. Bag 7 was treated with 0.01% DMSO alone, similar to the concentration of DMSO in the rest of PCs, and was considered as the control unit.

After gentle massaging, all bags were placed at 4 °C and stored undisturbed for up to 15 days. At selected storage intervals, 0.5 ml aliquots of PCs were aseptically removed from each bag, allowed to come back to room temperature, and assessed for platelet counts using an electronic cell counter (Coulter MAX-M, IZASA, Barcelona, Spain). Our next six different pools of PCs assessed the optimal concentration of those components that provided the highest maintenance of platelet counts during storage at 4 °C. These experiments were essentially performed as described above. Each bag contained 50 ml from nine pooled and filtered PCs, and the following reagents: bag 1, 0.50 mM amiloride; bag 2, 0.125 mM amiloride; bag 3, 100 μM SNP; bag 4, 25 μM SNP; bag 5, 0.25 mM amiloride + 50 μM SNP; bag 6, 0.50 mM amiloride + 100 μM SNP; bag 7, 0.125 mM amiloride + 25 μM SNP; bag 8, ThromboSol; bag 9, 0.01% DMSO.

The final experiments were carried out to assess the in vitro functional status of PCs supplemented with either ThromboSol, or with the simple combination of 0.125 mM amiloride and 25 μM SNP. In these sets of experiments, a pool of three ABO isogroup PCs was prepared, filtered, and distributed in three bags of 50 ml volume. One bag was supplemented with 0.125 mM amiloride + 25 μM SNP, and the other two either with ThromboSol, or with 0.01% DMSO. PCs were placed at 4 °C and stored as above without shaking. At days 1, 5, 10 and 15 of storage, 7 ml aliquots were removed from PCs and analysed for the following parameters: platelet count; platelet surface expression of GPIIb/IIIa, P-selectin (CD62P), and granulophysin (CD63); HSR; aggregation profile in response to several agonists; supernatant levels of lactic dehydrogenase (LDH), glucose, bicarbonate and lactate levels; pH; and concentrations of second messengers [cAMP, cGMP, and thromboxane B2 (TxB2)].

**Test and assays**

**Cell counts**

Cell counts in the PCs were determined with an electronic particle counter (STKS; Coulter Electronics, Hialeah, FL, USA).

**Flow cytometric analysis**

Flow cytometric analysis of platelet surface expression of P-selectin, CD63, GPIIb and GPIIb/IIIa was assessed in PC samples previously diluted (1 : 25) with 20 mM HEPES and 0.15 mM NaCl, pH 7.35. These experiments were carried out essentially as detailed elsewhere (Lozano et al., 1997), using the appropriate GP mAbs among those listed above, and a fluorescence flow cytometer (FACScan; Becton Dickinson, Mountain View, CA, USA). For each sample run, data acquisition of 10 000 an event was gated on forward and side-angle light scatter with gains adjusted to include the platelet population. Then, the fluorescence of stained platelets was analysed (CellQuest; Becton Dickinson) to obtain both the percentage of positively stained cells and the mean fluorescence intensity (MFI).

Fibrinogen binding to platelets was assessed by means of fluorescence-activated flow cytometry, as previously described with few modifications (Faraday et al., 1994). Briefly, prostaglandin E (50 ng/ml, final concentration) was added to PC aliquots. After centrifugation (800 × g for 8 min) the platelet pellet was rinsed three times in Tyrode’s buffer (138 mM NaCl, 2.9 mM KCl, 12 mM NaHCO3, 0.36 mM Na2HPO4, 5.5 mM glucose, 1.8 mM CaCl2, pH 7.4), and finally resuspended in the same buffer. Platelets (1 × 10^6) were incubated with 400 μg/ml fluor 488* fibrinogen (Molecular Probes, Eugene, OR, USA) and 50 μM thrombin receptor agonist (TRAP 14-mer; Sigma-Aldrich, St Louis, MO, USA) for 20 min. Acquisition and processing of data was carried out on a FACScan cytometer.

**Platelet morphological changes and aggregation studies**

Aggregation studies: To analyse the aggregation profile, samples were first centrifuged at 800 × g for 8 min and resuspended in AB plasma to a final concentration of 300 × 10^9/l. The aggregation response of platelets to ADP + adrenaline (10 μM each), ristocetin (1 mg/ml), and TRAP (50 μM) was monitored optically on an aggregometer (Aggrecoeur II, Menarini Diagnostics, Florence, Italy) set at 37 °C and 1000 rpm. Results were reported as the maximum change in light transmission (%) for a total time of 5 min, using the initial platelet suspension as the baseline and platelet poor plasma (PPP) as 100%.

Hypotonic shock response: For testing the HSR, platelets were adjusted to a concentration of 300 × 10^9/l as for aggregation studies, were mixed with distilled water (1.5 : 1 volume ratio of PCs : water) in a spectrophotometer cuvette. The optical density (OD) at 610 nm was recorded immediately after water addition (highest transmittance or lowest optical density) and following incubation of cells for 15 min, by means of a U-2000 spectrophotometer.
(Hitachi Ltd, Tokyo, Japan). An identical OD recording was made in parallel in adjusted PCs mixed with isotonic phosphate-buffered saline (PBS) instead of with water. The percentage HSR was determined by applying the formula 
\[
\frac{(\text{OD post 15 min in water} - \text{lowest OD})}{(\text{OD in PBS} - \text{lowest OD})} \times 100.
\]

**Biochemical studies**

Glucose and LDH concentrations in PPP samples were assessed by using a biochemical analyzer (RA-1000, Technicon, Tarrytown, NY, USA). Lactate content was measured by a standard enzymatic method (Sigma-Aldrich Quí´mica S.A., Madrid, Spain).

The pH and plasma bicarbonate levels were measured in a blood-gas analyzer (Radiometer ABL5, Copenhagen, Denmark).

The cAMP and cGMP levels in PC samples were determined by using commercially enzyme immunoassay (EIA) kit systems (RPN 225 and RPN 226, respectively; Amersham Pharmacia Biotech Ltd, Buckinghamshire, UK), following the manufacturer’s instructions.

The TxB₂ level was measured in PPP derived from PCs samples by an EIA kit system (RPN 220, Amersham Pharmacia Biotech Ltd), as indicated by the manufacturer.

**Statistical analysis**

All data are reported as mean ± SD from the results achieved in six different pools of PCs. Statistical differences between PC units, were tested by ANOVA, or by Wilcoxon’s tests using a computer program (StatView; Abacus Concepts, Calabasas, CA, USA). Differences were considered significant when \( P < 0.05 \).

**Results**

**Effect of ThromboSol reagents on platelet count changes during cold storage of PCs**

Cold temperatures expose fibrinogen-binding sites as a consequence of the activation process, leading to spontaneous aggregation of the cells that can be ultimately measured by a decrease in the cell number. The individual capacity of ThromboSol reagents to diminish the cold-induced loss of platelets was assessed in six different pools of PCs, in which PCs supplemented with each individual ThromboSol component were evaluated for changes in their platelet counts throughout refrigerated storage for up to 15 days. Table 1 summarizes the progressive loss of platelet counts displayed by either group of PCs during refrigerated storage. As shown, the lowest decrease was observed in units supplemented with ThromboSol, followed by those treated with either amiloride (0.25 mM) or SNP (50 μM). Thus, at the end of the established cold storage (15 days), only PCs treated with those agents displayed significantly higher platelet counts than control units treated with DMSO alone.

**Table 1. Changes in platelet counts in refrigerated PCs**

<table>
<thead>
<tr>
<th></th>
<th>Day 5</th>
<th>Day 10</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 mM amiloride</td>
<td>0.91 ± 0.07*</td>
<td>0.81 ± 0.07*</td>
<td>0.74 ± 0.09*</td>
</tr>
<tr>
<td>50 μM SNP</td>
<td>0.89 ± 0.06</td>
<td>0.74 ± 0.08</td>
<td>0.70 ± 0.09*</td>
</tr>
<tr>
<td>0.1 mM adenosine</td>
<td>0.86 ± 0.07</td>
<td>0.68 ± 0.08</td>
<td>0.61 ± 0.07</td>
</tr>
<tr>
<td>40 μM dipyridamole</td>
<td>0.81 ± 0.07</td>
<td>0.60 ± 0.06</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>0.2 mM quinacrine</td>
<td>0.83 ± 0.05</td>
<td>0.69 ± 0.08</td>
<td>0.61 ± 0.10</td>
</tr>
<tr>
<td>ThromboSol</td>
<td>0.95 ± 0.06*</td>
<td>0.89 ± 0.08*</td>
<td>0.77 ± 0.10*</td>
</tr>
<tr>
<td>Control (0.01% DMSO)</td>
<td>0.84 ± 0.07</td>
<td>0.67 ± 0.06</td>
<td>0.59 ± 0.07</td>
</tr>
</tbody>
</table>

Values are the percentage of platelet count after the specified storage period, compared with that at day 1 which was considered as 100%.

Mean (±SD) platelet count at day 1 was 1037.67 ± 274.38 × 10³/l (n = 6).

*P < 0.05 vs. control platelet concentrate (PC), at each specific day of storage.

SNP, sodium nitroprusside; DMSO, dimethyl sulphoxide.

**Dose-dependent effect of amiloride and SNP in the maintenance of platelet counts during refrigerated storage**

In order to find out the optimal dose of both amiloride and SNP conferring a protection from cold-induced platelet loss, we analysed six new different pools of PCs supplemented with these reagents at either half or double dose than that included in ThromboSol. In these experiments, we found that supplementation of PCs with a combination of 0.125 mM amiloride and 25 μM SNP resulted in maintenance of platelet counts throughout storage, similar to that displayed by units treated with ThromboSol (Table 2) (78% vs. 80%, at day 15, respectively; \( P > 0.05 \)). Synergism between those agents was observed as their use in combination provided a slightly higher protection from cold-induced platelet loss than their single use at identical concentrations (73% and 70% recovery at day 15 for amiloride and SNP, respectively). Intriguingly, a shift from beneficial to deleterious effect was observed if the dose of these drugs when used individually or combined, doubled that included in ThromboSol (Table 2).
Quality assessment of refrigerated PCs supplemented with a reduced dose of amiloride and SNP

Once preliminary experiments demonstrated that a combination of 0.125 mM amiloride and 25 μM SNP had a protective effect against decrease of platelet counts during refrigerated storage of PCs comparable with ThromboSol, we designed a set of six new assays aiming to assess the in vitro quality of PCs supplemented with that combination of platelets inhibitors.

When the quality of PCs is evaluated on a routine basis, the use of in vivo assays is clearly not practical, and in vitro tests are required (Murphy et al., 1994). As the release reaction during storage of PCs increases expression on the platelet surface of GPs from the granule membranes, indices widely used for the evaluation of quality of PCs include the platelet surface expression of P-selectin (CD62P or GMP 140, from α-granules and dense granules), and granulophysin (CD63, from dense granules and lysosomes). As shown in Table 3 storage under refrigerated conditions promoted a rise in the activation markers of platelets in both treated and control PCs, as assessed by determining the rise during storage in the percentage of platelets positive for the surface markers CD62P and CD63. The supplementation with the different agents had no apparent effect on the expression of these markers, as this increase was similar by the end of storage in both amiloride + SNP and ThromboSol-treated units, compared with that in parallel untreated PCs.

As the haemostatic effectiveness of platelets is related to the ability of the membrane GP Ibβ and -IIb/IIIa complex to interact with their respective physiological ligands (mainly von Willebrand factor and fibrinogen, respectively), an evaluation of the immunological levels and functional integrity of these receptors was performed by flow cytometry using the appropriate mAb or fluorochrome-labelled fibrinogen, and by platelet aggregation following stimulation with ristocetin or with physiological agonists. While a similar expression of GPIb/IIIa was assessed under the three study conditions, the GPIbα expression was significantly better preserved in units supplemented with amiloride + SNP or with ThromboSol, than that in paired control units on days 10 and 15 of storage (Table 3). As for the aggregation response and fibrinogen-binding, storage induced a similar decrease under the three conditions tested (Figure 1, Panels a and b, and Table 4). We also found a similar and mild cold storage-promoted reduction in the response to 1 mg/ml ristocetin in both control units and PCs treated with either ThromboSol or with amiloride + SNP (Figure 1, panel c), having little impact in this response the slightly better preservation of GPIbα levels in PCs supplemented with amiloride + SNP. Similarly, during preparation and during storage of PCs, platelets are exposed to a variety of mechanical and chemical influences that may promote the platelet shape transformation. As shown in Figure 1 (panel d), storage decreased the ability of platelets to recover from hypotonic shock, but such ability was slightly, but not significantly, better preserved in treated PCs, specially in those supplemented with amiloride + SNP (47% recovery vs. 40% in ThromboSol-units, and 38% in control-PCS at day 15).

In parallel to the expanding activation, the inevitable ageing and the progressive metabolic depletion of PCs during storage impairs the barrier function of the plasma membrane, leading to swelling of the platelets and

| Table 2. Dose-dependent effect of amiloride + SNP combination in platelet counts during cold storage of PCs |
|-----------------|----------------|----------------|----------------|
|                 | Day 5          | Day 10         | Day 15         |
| 0.50 mM amiloride| 0.87 ± 0.04    | 0.77 ± 0.12    | 0.58 ± 0.12    |
| 0.125 mM amiloride| 0.91 ± 0.03*   | 0.85 ± 0.03*   | 0.73 ± 0.04    |
| 100 μM SNP       | 0.86 ± 0.04    | 0.79 ± 0.07    | 0.70 ± 0.04    |
| 25 μM SNP        | 0.88 ± 0.06    | 0.79 ± 0.07    | 0.70 ± 0.06    |
| 0.25 mM amiloride + 50 μM SNP | 0.92 ± 0.05 | 0.87 ± 0.07 | 0.75 ± 0.06 |
| 0.50 mM amiloride + 100 μM SNP | 0.88 ± 0.06 | 0.77 ± 0.14 | 0.58 ± 0.18 |
| 0.125 mM amiloride + 25 μM SNP | 0.93 ± 0.01* | 0.86 ± 0.03* | 0.78 ± 0.04* |
| ThromboSol      | 0.92 ± 0.02*   | 0.88 ± 0.04*   | 0.80 ± 0.07*   |
| Control (0.01% DMSO) | 0.85 ± 0.04 | 0.75 ± 0.05 | 0.67 ± 0.05 |

Values are given as percentage of platelet count after the specified storage period, compared with that at day 1 which was considered as 100%.

Mean (±SD) platelet count at day 1 was 1216.42 ± 339.35 × 10⁹/l (n = 6).

*P < 0.05 vs. control PC at each specific day of storage.

SNP, sodium nitroprusside; PC, platelet concentrate; DMSO, dimethyl sulphoxide.
eventually to lysis. Assessing cell death can be performed by the supernatant level of LDH activity as a marker of cell lysis in PCs. We observed that in all units tested, storage promoted a rise in the cell lysis marker, LDH. As expected, this increase was significantly lower in treated PCs, either treated with amiloride + SNP or with ThromboSol, when compared with control units containing DMSO alone (Table 5).

During storage of PCs, both glycolysis and oxidative phosphorylation fuel regeneration of ATP avoid energy depletion. Platelets have a prominent glycolytic rate, resulting in substantial glucose depletion and lactate production. A fall in the pH occurs when the buffering capacity provided by bicarbonate is consumed. In our conditions, these inhibitors had a minor influence in the slow metabolic rate of PCs during refrigerated storage, as no differences were observed between treated and untreated control PCs regarding their metabolic behavior during the cold storage (similar pH, glucose, lactate and bicarbonate levels) (Table 5).

The ongoing metabolic activity during storage may also alter the platelet balance in second messengers, the natural regulators of platelet activation. Cyclic nucleotides, mainly cAMP and cGMP, are inhibitors of this process, whereas other agents such as TxA2 are activators (levels increase throughout storage) (Rivera et al., 1999). As expected by the presence of cAMP effectors (adenosine and dipyridamole), a significantly higher level of this second messenger was found by the end of storage in ThromboSol units (Figure 2, panel a). While the cAMP level in amiloride + SNP PCs was similar to that in control units, the presence of SNP led to significantly higher content of cGMP, although this level of cGMP was significantly lower than that in units supplemented with ThromboSol (Figure 2, panel b). Although we observed no significant differences in the supernatant levels of TxB2 among treated and untreated units (Figure 2, panel c), by the end of the storage (day 15), the ratio between TxB2 level (potentially promoting activation) and cGMP (potentially inhibiting activation) was significantly higher in control PCs than in those supplemented with amiloride + SNP or with ThromboSol (ratio values of 12.8, 4.2, and 2.3, respectively) (Figure 2, panel d).

**Discussion**

We and other groups have previously evaluated the refrigerated storage of PCs supplemented with ThromboSol, a mixture of second messenger effectors, as a potential strategy for storage of PCs beyond the current 5-day limit. Considering that the cold storage lesion somehow
resembles the platelet activation with agonists, and thus may be modulated by platelet inhibitors, in vitro studies have demonstrated that the pharmacological supplementation of PCs with ThromboSol, significantly favours the maintenance of the in vitro integrity and responsiveness of platelets during extended storage at refrigerated temperature (Connor et al., 1996; Lozano et al., 1999, 2000; Rivera et al., 1999; Currie et al., 1999; Pedrazzoli et al., 2000). However, the pharmacological load of ThromboSol is high, containing reagents such as ticlopidine that would unlikely have any beneficial effect when used in vitro (Saltiel & Ward, 1985). Thus, this study was undertaken to assess the individual capacity of each ThromboSol reagent to prevent cold-induced decrease in platelet counts, and to compare the in vitro properties of PCs supplemented with ThromboSol with those of PCs treated with a simpler, pharmacologically reduced, combination of platelet inhibitors.

Figure 1. Changes in aggregation profile (percentage light transmission) and in hypotonic shock response (HSR) in control and treated platelet concentrates (PCs) during refrigerated storage. The aggregation response to adenosine diphosphate (ADP) + adrenaline (EPN) it was evaluated as percentage of change vs. the initial response of PCs at day 1 within each experiment. Data are mean ± SD (n = 6).
Table 4. Fibrinogen binding after TRAP stimulation (50 μM) in 25 μM SNP + 0.125 mM amiloride-treated PCs, ThromboSol-treated units, and control PCs, during refrigerated storage

<table>
<thead>
<tr>
<th></th>
<th>Day 1 Initial platelet pool</th>
<th>Day 5 SNP + amiloride</th>
<th>Day 5 ThromboSol</th>
<th>Day 5 Control</th>
<th>Day 10 SNP + amiloride</th>
<th>Day 10 ThromboSol</th>
<th>Day 10 Control</th>
<th>Day 15 SNP + amiloride</th>
<th>Day 15 ThromboSol</th>
<th>Day 15 Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen binding percentage of positive platelets</td>
<td>47.42 ± 26.8</td>
<td>35.9 ± 18.8</td>
<td>32.5 ± 28.8</td>
<td>31.9 ± 23.4</td>
<td>12.4 ± 11.1</td>
<td>20.0 ± 19.4</td>
<td>19.0 ± 11.5</td>
<td>8.9 ± 7.3</td>
<td>7.0 ± 7.9</td>
<td>10.6 ± 3.6</td>
</tr>
</tbody>
</table>

Data are mean ± SD (n = 6).

TRAP, thrombin receptor agonist; SNP, sodium nitroprusside; PC, platelet concentrate.

Table 5. Metabolic and integrity parameters in 25 μM SNP + 0.125 mM amiloride-treated PCs, ThromboSol-treated units, and control PCs, during refrigerated storage

<table>
<thead>
<tr>
<th></th>
<th>Day 1 Initial platelet pool</th>
<th>Day 5 SNP + amiloride</th>
<th>Day 5 ThromboSol</th>
<th>Day 5 Control</th>
<th>Day 10 SNP + amiloride</th>
<th>Day 10 ThromboSol</th>
<th>Day 10 Control</th>
<th>Day 15 SNP + amiloride</th>
<th>Day 15 ThromboSol</th>
<th>Day 15 Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.32 ± 0.57</td>
<td>7.26 ± 0.03</td>
<td>7.28 ± 0.04</td>
<td>7.29 ± 0.05</td>
<td>7.21 ± 0.04</td>
<td>7.23 ± 0.03</td>
<td>7.23 ± 0.04</td>
<td>7.13 ± 0.08</td>
<td>7.16 ± 0.07</td>
<td>7.16 ± 0.05</td>
</tr>
<tr>
<td>Bicarbonate (mm)</td>
<td>15.5 ± 0.5</td>
<td>11.8 ± 0.7</td>
<td>12.2 ± 0.7</td>
<td>12.0 ± 1.5</td>
<td>9.2 ± 0.4</td>
<td>9.8 ± 0.4</td>
<td>9.5 ± 0.8</td>
<td>6.5 ± 0.7</td>
<td>7.8 ± 0.7</td>
<td>7.3 ± 1.0</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>342.8 ± 11.9</td>
<td>306.4 ± 9.0</td>
<td>310.2 ± 13.7</td>
<td>312.2 ± 9.2</td>
<td>279.7 ± 16.0</td>
<td>284.0 ± 10.8</td>
<td>284.2 ± 16.7</td>
<td>250.2 ± 8.9</td>
<td>261.5 ± 19.7</td>
<td>259.2 ± 11.8</td>
</tr>
<tr>
<td>Lactate (mm)</td>
<td>4.2 ± 0.5</td>
<td>8.3 ± 1.2</td>
<td>7.8 ± 0.6</td>
<td>7.6 ± 0.9</td>
<td>10.5 ± 1.2</td>
<td>10.1 ± 1.1</td>
<td>9.8 ± 0.6</td>
<td>14.0 ± 2.0</td>
<td>13.5 ± 0.9</td>
<td>13.5 ± 1.9</td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>264.3 ± 16.4</td>
<td>276.7* ± 14.1</td>
<td>279.5 ± 12.6</td>
<td>284.7 ± 9.0</td>
<td>305.2* ± 16.3</td>
<td>305.2* ± 31.1</td>
<td>333.8 ± 18.2</td>
<td>331.2* ± 19.8</td>
<td>341.7* ± 33.4</td>
<td>382.3 ± 11.3</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD (n = 6).

*P < 0.05 vs. control PC at each specific day of storage.

SNP, sodium nitroprusside; PC, platelet concentrate; LDH, lactate dehydrogenase.
It is well-known that the rate of appearance of the platelet storage lesion (PSL) in PCs can be influenced to a variable degree by several physical, chemical, and metabolic factors related to platelet withdrawal, preparation, and storage. A wide number of in vitro tests are available for routine monitoring of the extent of PSL, although a universally accepted and standardized protocol is still lacking. One of the final stages of cold-induced alterations in platelets is the spontaneous aggregation, leading to a decrease in the cell counts. We used this parameter to assess the degree of cold-induced effects on stored platelets. Our first set of studies shows that amiloride and SNP are the most efficient ThromboSol reagents in preventing the reduction of platelet counts in refrigerated PCs. When

Figure 2. cAMP, cGMP and thromboxane B$_2$ (TxB$_2$) levels in refrigerated control and treated platelet concentrates (PCs). Data are mean ± SD (n = 6). *P < 0.05 vs. control (0.01% dimethyl sulphoxide, DMSO) at each studied day of storage.
used in combination, they provide a protective effect against platelet loss similar to ThromboSol, even if their dose is half-reduced compared with that of ThromboSol. Amiloride, an inhibitor of the plasma membrane Na⁺/H⁺ pump, inhibits platelet aggregation and secretion induced by thrombin, collagen, platelet-activating factor and adrenaline (Siffert, Gegenbach & Scheid, 1988), decreases agonist-induced shape change, possibly by interfering with cytoskeletal assembly (Siffert & Akkerman, 1988), and inhibits monocyte adhesion to storage bags facilitating reduction in cytokine production (Wood et al., 1999). On the contrary, SNP is a drug that activates the guanylate cyclase to generate cGMP, a second messenger that inhibits platelet activation. Sodium nitroprusside exhibits both in vivo and in vitro antiplatelet effects, as assessed by inhibition of subsequent ADP-induced aggregation (Chirkov et al., 1991).

In our study, the assessment of multiple platelet parameters show that refrigerated PCs supplemented with the simple combination of amiloride and SNP, at half concentration to that in ThromboSol, not only prevents platelet aggregation at a similar degree than ThromboSol, but also display in vitro characteristics similar to that of ThromboSol-treated PCs, showing significantly better features than untreated control units. As for ThromboSol, treatment of PCs with amiloride + SNP is unable to fully abrogate the cold-induced activation process, as still some markers, such as P-selectin, which does not uniformly predict platelet viability (Berger, Hartwell & Wagner, 1998) are similarly expressed than in control units. Our study shows that platelet surface expression of GP Ibα is better preserved in refrigerated PCs treated either with amiloride + SNP or with ThromboSol than in untreated units. A recent study has shown that the cold-induced clustering of the platelet GP Ibα is not prevented by cytoskeleton inhibitors (Hoffmeister et al., 2003a), and that glycosylation of the receptor restores survival of chilled blood platelets (Hoffmeister et al., 2003b), but the effect of these second messenger effectors in this context remains to be elucidated.

Evaluation of the disk to sphere transformation has been shown to be a useful predictor of in vivo platelet viability (Seghatchian & Krailadsiri, 1997). In addition to shape transformation, another index of platelet activation is the assessment of the ability of major adhesive receptors, such as GPIIb or GPIIb/IIIa to bind physiological ligands in response to appropriate stimuli. The overall in vitro quality of treated and untreated PCs showed no differences in the ability to change shape (HSR), or in the response to different agonists (percentage aggregation induced by ristocetin, ADP + adrenaline or TRAP, and binding of labelled fibrinogen induced by TRAP activation) over the storage of 15 days. Moreover, the supplementation of PCs with second messenger effector (amiloride + SNP or ThromboSol) led to lower levels of LDH released into the medium throughout cold storage, suggesting a reduced lysis of cells compared with control units.

Measurement of cyclic nucleotides showed that the effect of these agents during cold storage might be related with maintaining a better balance between second messengers inhibiting and promoting platelet activation, cGMP and Tx A₂, respectively. Collectively, these data show that the attenuated decrease of the platelet counts with the addition of second messenger effectors might be a consequence of both (1) reduced lysis of the cells and (2) decreased spontaneous aggregation because of the diminished increase in the TxB₄ : cGMP ratio.

Overall, our results indicate that the presence of all reagents that make up ThromboSol is unnecessary to provide its claimed beneficial effect on the in vitro quality of refrigerated PCs. Noteworthy, an alternative ThromboSol solution containing only 0.25 mm amiloride; 0.1 mm adenosine; 50 μm SNP, has been used in combination with 2% DMSO to preserve platelets during cryopreservation (Lozano et al., 1999, 2000; Currie et al., 1999; Pedrazzoli et al., 2000). As far as we know, no clinical studies have been performed evaluating the clinical effectiveness of refrigerated PCs treated with full ThromboSol, and only in few clinical studies, washed autologous PCs cryopreserved with 2% DMSO and ThromboSol-containing amiloride-adenosine-SNP have been transfused (Currie et al., 1999; Pedrazzoli et al., 2000). For a potential clinical use of chemically treated platelet products, the addition of the lowest possible pharmacological load seems desirable. Therefore, treatment of refrigerated PCs solely with amiloride + SNP may be a better option than with ThromboSol, as they provide a similar protection of the in vitro platelet function.

In summary, our study supports that supplementation of PCs with reduced doses of amiloride and SNP (0.125 mm and 25 μm) appears to be as efficient as ThromboSol in preventing the reduction in platelet counts of PCs stored at 4 °C, and in ameliorating the decreased in vitro functionality of refrigerated PCs compared with untreated units. The reduced pharmacological load of the former treatment argues in favour of its eligibility when designing strategies for cold storage of PCs.

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References


