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Effect of cold-storage on the accumulation of bioreactive substances in platelet concentrates treated with second messenger effectors

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Background and Objectives. The mandatory 5-day of shelf-life platelet concentrates (PCs) creates outdating and inventory control problems in blood banking. Moreover, storage of PCs at 22-24ºC has been associated with a time-dependent accumulation of pyrogenic cytokines, potentially harmful for recipients. Previous studies have shown that supplementation of PCs with ThromboSol, a mixture of second-messengers effectors, might allow storage of functionally active platelets at refrigerated temperature to be extended. This study further investigates this storage approach by comparing the accumulation of bioactive compounds in standard and refrigerated PCs.

Design and Methods. The PCs were supplemented with ThromboSol or a control solution and stored in parallel at 22ºC with continuous agitation or undisturbed at 4ºC. Samples were removed on days 1, 5, 9 of storage, and assayed for their content of interleukin (IL)-6, IL-8, tumour necrosis factor (TNF)-α transforming growth factor (TGF)-β1, and anaphylatoxins C3a and C4a.

Results. Throughout storage, refrigerated PCs, both ThromboSol-treated and untreated units, displayed a slightly lower level of IL-6 and significantly lower concentration of IL-8 than conventionally stored PCs. ThromboSol slightly reduced the level of these cytokines in PCs. Throughout storage at 22ºC, an accumulation of anaphylatoxins C3a and C4a was seen both in both control and ThromboSol-treated PCs. This accumulation was significantly reduced in control PCs stored at 4ºC, but not in refrigerated PCs supplemented with ThromboSol. Cold-storage, with or without ThromboSol, had a minor effect on the accumulation of TGF-β1 in PCs.

Interpretation and Conclusions. Our data confirm that release of bioactive compounds during in vitro storage of PCs is a temperature-sensitive process. The ThromboSol-refrigeration system could be a useful alternative for extending storage of PCs, without increasing the accumulation of cytokines (IL-6, IL-8), known to be involved in febrile reactions in recipients. Nevertheless, this storage system has no benefit on the level of other bioactive compounds (TGF-β1, anaphylatoxins C3a and C4a) in PCs.

Key words: refrigerated PCs, storage, cytokines, anaphylatoxins, ThromboSol

The use of platelet transfusion has increased considerably over the last three decades, in part due to the increasing number of cardiac, liver, and bone marrow transplants and in part due to the extended use of intensive myelosuppressive chemotherapy for malignant conditions.[1] At present, allogeneic platelet concentrates (PCs) stored in the liquid state at 22ºC, are the most prevailing platelet product being employed.

Despite the considerable advance that has been made in the preparation and preservation of current PCs, with the development of new isolation procedures and more effective storage containers, transfusion of these products is still associated with certain risks for recipients. These risks are transmission of infections, alloimmunization resulting in refractoriness, immunomodulation, graft-versus-host disease, and febrile non-hemolytic transfusion reactions (FNHTRs) caused by bioreactive substances accumulated in the product during storage.[2] Moreover, current PCs have the important shortcoming of having a mandatory shelf-life of five days. This is largely dictated by the high risk of bacterial contamination and because of unacceptable platelet lesions when storage at 22ºC is prolonged beyond 5 days.[3,4] This short shelf-life leads to the outdating of a significant number of units, and creates a considerable inventory control problem for blood banks.

With the goal of producing hemostatically active platelet products with a longer shelf-life, several experimental approaches have been explored; i.e. refrigeration, cryopreservation, lyophilization, and artificial platelet substitutes.[5] Among these, storage in the liquid state at 4ºC, may be the simplest and least expensive approach, since it would not involve substantial modification of current methods for preparing PCs, and requires no special equipment. In addition, refrigerated storage of PCs may achieve two desirable goals. The first of these goals is inhibition of the growth of bacterial pathogens potentially present in PCs, thus allow-

ing the possibility of prolonging the shelf-life of PCs without increasing the risk of platelet transfusion-associated bacteriemia.[6] The second goal is to improve cellular metabolism, and hence impair the production of pyrogenic cytokines by passenger leukocytes during storage of PCs and, subsequently, reduce the risk of FNHTRs in recipients of PCs. Indeed, a recent study has shown that interleukin (IL)-2 and IL-6 secretion by mononuclear leukocytes is significantly lower at 22ºC than at 37ºC, suggesting that this process is strongly influenced by temperature.[7] Moreover, a previous work has shown that Pcs, stored at 4ºC in the presence of ThromboSol – a combination of second messenger effectors - displayed inhibited bacterial growth and reduced accumulation of the leukocyte-derived cytokines IL-6, IL-1β and tumour necrosis factor α (TNF-α).[8]

Refrigerated storage of platelets had been abandoned because platelets were found to be significantly and irreversibly affected by low temperatures, an effect referred to as *cold storage lesion*.[6,9,10] Moreover, recent advances in understanding the mechanism of cold temperature effects on platelets have led to novel strategies to prevent these effects from occurring and have resurrected the possibility of liquid cold storage of PCs. Such strategies include physical methods, and the use of antifreeze glycoproteins, cytoskeletal stabilizers, or signal transduction inhibitors.[6] In this sense, we and others have shown that supplementation of PCs with ThromboSol - a combination of second-messenger effectors - provides a protective effect that allows prolonged storage of PCs at 4ºC without significant loss of cells or cell function.[11-14] This storage solution has also demonstrated to be useful for platelet cryopreservation.[14-19]

In order to provide furthers insights into the benefits of ThromboSol-assisted cold storage of PCs, we compared the accumulation of bioreactive substances in PCs supplemented or not with this combination of second-messengers, and stored at either 4ºC or under conventional blood banking conditions.

Design and Methods

Materials

ThromboSol, containing amiloride, adenosine, sodium nitroprusside (SNP), dipyridamole, ticlopidine, and quinacrine, was kindly provided by LifeCell Corporation (Woodlands, TX, USA) as a 500-fold concentrate in dymethylsulphoxide (DMSO). Immediately before its use, a 50 × working solution was made in sterile phosphate-buffered saline.

Preparation, storage, and sampling of PCs

Whole blood (450 mL) from repeat blood donors was collected into a triple-bag system (Karmi series, Kawasumi Lab, Tokyo, Japan), in which the primary pack contains 63 mL of CPD. The platelet container is made of polyvinylchloride and, according to the manufacturer, has adequate gas exchange properties. This plastic bag has been licensed in the European Union for the storage of platelets for up to 5 days, according to the European Union Directive for Medical Devices (93/42/EEC).

PCs were obtained from whole blood as previously described (20). In each series of experiments, five ABO-compatible PCs were pooled using an sterile device (SCD, Haemonetics, Braintree, MA, USA), and redistributed into four bags (Kawasumi Lab Inc, Tokyo, Japan) of 50 mL volume. Upon preparation there were no significant differences in cell counts between the four bags, each containing $1,116\pm217\times10^9$ platelets/L and $1.32\pm0.31\times10^9$ leukocytes/L. Two bags were injected with 1 mL of 50 × ThromboSol solution via a sampling site coupler (Baxter SA, Barcelona, Spain) to achieve the following concentrations of ThromboSol reagents: 0.25 mM amiloride, 0.1 mM adenosine, 50 µ SNP, 40 µ dipyridamole, 0.75 m ticlopidine, 0.2 µ quinacrine, and 0.2% DMSO. The remaining two bags received phosphate-buffered saline and were considered control units. After gentle massaging, ThromboSol-treated and control units were placed in pairs, either on a horizontal agitator (60 cycles/min) at 22°C, or at 4°C without shaking. At selected intervals of storage, 7 mL aliquots of control and treated PCs were aseptically removed and centrifuged (12,000 × g for 5 min) to obtain platelet poor plasma (PPP) samples that were stored at –80ºC until analysis.

Measurement of bioreactive substances in PCs

The concentrations of IL-6, IL-8, TNF-α and transforming growth factor α1 (TGFβ1), were measured in PPP samples by enzyme-linked immunosorbent assays (ELISA) (Amersham Pharmacia Biotech, Barcelona, Spain), according to the manufacturer's instructions. The sensitivity of these assays was found to be 0.6, 25, 0.3 and 15 pg/mL for IL-6, IL-8, TNF-α and TGF-β1, respectively. The levels of the anaphylatoxins C3a and C4a were quantified by using C3a-desArg and C4a-desArg [^{125}I] assay systems (Amersham Pharmacia Biotech). When necessary, PPP samples were appropriately diluted with a solution recommended by the manufacturer before measurement of bioactive substances. Samples showing levels below the sensitivity of the assays were scored as containing the lowest concentration detectable by the assay.

Statistical analysis

Concentrations of IL-6, IL-8, TGF-β1 in PCs are given as mean ± SD of the values found in five to six series

of experiments performed with different platelets. The normal distribution of data was evaluated using NCSS 6.0 software. Statistical differences between the level of cytokines in control and ThromboSol-treated PCs throughout the storage period were tested by the paired *t* test (IL-8 and TGFβ1) or Wilcoxon's signed rank test (IL-6), using the StatView computer program (Abacus Concepts, Calabasas, CA, USA). Differences were considered significant when $p<0.05$. The concentrations of anaphylatoxins (C3a-desArg and C4a-desArg) were measured in a pool of six samples drawn from units at days 1, 5 and 9 of storage.

Results

Effect of thromboSol and refrigerated storage of PCs on the accumulation of IL-6 and TNF-α

Table 1 shows the IL-6 levels in the plasma supernatant of control and ThromboSol-treated PCs during storage at either 22ºC or 4ºC.

Despite the low levels of IL-6, we found that control PCs stored at 22ºC tended to accumulate this cytokine during storage. By contrast, refrigerated PCs, either ThromboSol-treated or control units, showed a slight decline in the initial low content of IL-6 ($p=0.04$, levels at days 5 and 9 vs. day 1 contents). We observed that ThromboSol treatment by itself, independently of the temperature, reduced IL-6 accumulation in PCs. Thus, throughout storage ThromboSol-treated PCs stored at 22ºC displayed lower amounts of IL-6 than control units, and by the end of the storage (day 9) had a 3-fold lower concentration of IL-6 than the control PCs ($p=0.04$).

We also investigated the potential accumulation of TNF-α in PCs in every studied condition. Despite the use of a high sensitivity assay we did not find detectable levels of this cytokine at any time during storage in any of the PCs.

Effect of ThromboSol and refrigerated storage of PCs on the accumulation of IL-8

Table 1 summarizes the IL-8 levels in PCs throughout storage. At 22ºC, we appreciated a time-dependent accumulation of this cytokine both in ThromboSol-treated PCs and in control units. However, control PCs displayed significantly higher levels of IL-8 than treated-PCs (10-fold higher by day 9) so that, as above, the addition of ThromboSol, by itself, seemed to prevent the accumulation of IL-8 during the course of storage.

In contrast to units stored at 22ºC, refrigerated PCs displayed no detectable accumulation of IL-8 over the 9-day storage period (Table 1). This beneficial effect of cold storage was significant in both control and ThromboSol-treated PCs at every storage time analyzed.

Table 1. Levels of cytokines and chemokines in control (C) and ThromboSol-treated (T) PCs stored at either 22° or 4°C.

	(C)22º	*(C) 4º*	*(T) 22º*	*(T) 4º*
IL-6 (pg/mL)(n=5)				
Day 1	5.0±0.94	4.45±1.25	3.01#	4.64±1.03
Day 5	5.12±3.29	3.42±0.80*	4.18±1.76	3.52±0.85*
Day 9	6.56±6.96	3.04±0.54*	2.12±1.74°	2.95±0.46*
IL8 (pg/mL)(n=6)				
Day 1	272±77	47±12°	101±32°	47±12°
Day 5	2582±1612*	47±9°	310±89*°	46±11°
Day 9	4109±2192*	51±8°	389±99*°	50±11°
TGF-β1 (ng/mL)(n=5)				
Day 1	2.07±0.27	2.13±0.31	2.15± 0.24	2.70±0.70
Day 5	2.77±0.18*	2.66±0.76	2.93±0.12*°	2.44±0.47
Day 9	3.63±0.16*	3.21±0.34*	3.45±0.25*	3.26±0.90

**p<0.05 vs. Day 1 values for each storage condition. °p<0.05 vs. values of C22ºC at each storage time. #Value obtained in a pooled sample of five different PCs.*

Effect of ThromboSol and refrigerated storage of PCs on the accumulation of TGF-β1

All PCs, irrespectively of the storage conditions, displayed a storage-promoted rise in the level of the platelet-derived cytokine TGF-β1 (mean increase from 2.26±0.29 on day 1 to 3.38±0.19 on day 9) (ng/mL) ($p<0.001$) (Table 1). Nevertheless, the accumulation of TGF-β1 over the 9-day storage period was slightly less pronounced under refrigeration. Of note, the presence of ThromboSol had no significant effect on the level of TGF-β1 in PCs at either storage condition.

Effect of ThromboSol and refrigerated storage of PCs on the accumulation of anaphylatoxins

We investigated the time-dependent accumulation of anaphylatoxins C3a-desArg and C4a-desArg in the plasma supernatant of PCs. As shown in Figure 1, the C3a-desArg and C4a-desArg levels in control PCs stored under standard blood-banking conditions increased by 7-fold and 3-fold, respectively, during the 9-day storage period.

As for IL-6, IL-8 and TGF-β1, we found that refrigerated storage of control units without agitation led to lower accumulation of C3a and C4a (Figure 1). Of note, we also observed that, in contrast to findings for the above compounds, treatment of PCs with ThromboSol favored the accumulation of anaphylatoxins during storage at both 22 and 4ºC (Figure 1).

Discussion

The accumulation of cytokines and other bioactive compounds during storage of standard PCs has been

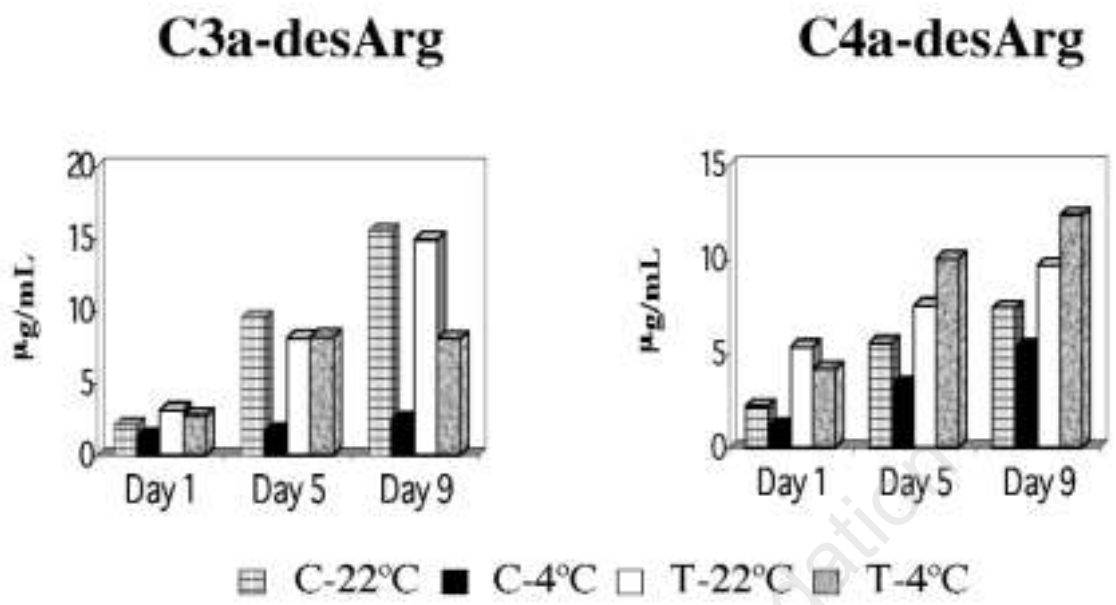

Figure 1. Plasma C3a-desArg and C4a-desArg contents in control (C) and ThromboSol-treated (T) PCs stored at 22ºC under standard blood banking conditions, or at 4ºC. Anaphylatoxin concentrations were determined in PC supernatant samples by radioimmunoassay, as described in Material and Methods. At each storage time and condition, columns represents the value of a sample from a pool of six different PCs.

related to the incidence of several adverse reactions in recipients.[2] Some of these bioactive compounds are thought to originate from the active metabolism of passenger leukocytes and other cells,[21-23] a process known to be temperature dependent. This prompted us to investigate the effect of cold storage on the accumulation of bioactive compounds in PCs. Being aware of the detrimental consequences of cold temperature on platelets, some of the studied PCs were supplemented with a mixture of second messenger effectors -ThromboSol-, which has been shown to protect platelets effectively from cold damage.[11-14]

We found that refrigeration significantly reduces the accumulation of two leukocyte-derived cytokines, IL-6 and IL-8 (2- and 80-fold, respectively, by the end of the storage). Interleukin-6 is a pyrogenic cytokine, and its level in PCs has been correlated with the frequency and severity of FNHTRs.[24,25] Chemokine IL-8, despite being non-pyrogenic, seems to play a major role in inflammatory responses, because of its neutrophil chemotactic- and activating-functions.[21,26] The mechanisms accounting for reduced cytokine concentrations in refrigerated PCs remain speculative. Federowicz *et al.* reported that IL-6 levels decreased during storage of leukocyte-reduced blood components. The suggested contributing factors to this finding were degradation of the cytokine throughout storage and lack of its production because of efficient pre-storage removal of white blood cells (WBC).[27] Yu *et al.*[28] have suggested that leukocyte secretion of IL-8 requires active protein synthesis, a temperature-modulated process that is significantly reduced at low temperatures. Thus, our current findings could reflect a cold-induced blockade of IL-6 and IL-8 production by leukocytes, and the progressive degradation of the existent cytokines during refrigerated storage of PCs.

We also observed that ThromboSol, potentially useful for protecting PCs from cold damage, provides, by itself, an inhibitory effect on IL-6 and IL-8 production. These results confirm and extend the recent findings of Currie *et al.*[8] and may suggest that some of the second messenger effectors and/or the 0.2% DMSO present in ThromboSol could modulate not only platelet activation but also WBC metabolism.

TGF-β1 is a platelet-derived cytokine that also accumulates during standard storage of PCs. Its role in platelet transfusion reactions remains unclear, but it may trigger inflammatory and immune responses by inducing secretion of cytokines (such as IL-1 and IL-6) and prostaglandins, and by upregulating IgA generation by activated B-lymphocytes.[29,30] In contrast to the finding concerning leukocyte-derived cytokines, i.e. IL-6

and IL-8, we observed that cold temperature has little influence on the storage-promoted accumulation of TGF-β1 in PCs. This finding was expected, since cold induces platelet activation,[9] and TGF-β1 is claimed to be a marker of platelet activation.[22] Treatment with ThromboSol displayed no protective effect on the accumulation of TGF-β1, despite this solution having been shown to inhibit platelet activation during refrigerated storage.[11] However, we have recently found that ThromboSol, while preserving the functionality of refrigerated platelets, is ineffective in inhibiting some cold and/or storage-promoted signs of platelet activation, such as P-selectin expression.[12]

Previous reports have suggested that complement activation in stored PCs is a potential pathogenetic mechanism of FNHTRs, by inducing the release of cytokines from monocytes.[23,31] In addition, anaphylatoxins are seen as mediators of inflammatory reactions, being involved in histamine release, prostaglandin and leukotriene generation, WBC chemotaxins, and platelet and WBC adhesion and aggregation.[32] As we expected from previous data from our laboratory[33,34] and from other investigators,[23,31] standard storage of PCs leads to release of anaphylatoxins. In contrast, PCs incubated undisturbed at 4ºC accumulated markedly less C3a and slightly less C4a. Whether this lower accumulation is due to cold temperature, to the lack of agitation, or to both factors, cannot be determined from our experimental design. Our study evidenced that the accumulation of C3a during the standard storage, as well as the reduction in C3a levels in refrigerated PCs by effect of cold, were more remarkable than those of C4a. This may reflect the different mechanisms of production of both products. In fact, the generation of C3a has been related to activation of the alternate pathway of the complement by contact of plasma with plastic surfaces, while accumulation of C4a might be interpreted as an indicator of the activation of the classical pathway of the complement cascade.[35] Some authors have suggested that activation of C4a is at least partially dependent on the degradation of leukocytes or platelets,[23] but this concept remains unclear.[33,34] An unexpected finding was that treatment with ThromboSol diminished the protective effect of cold on the accumulation of C3a and C4a in PCs. This may suggest that some of their components, including DMSO, may induce activation of complement pathways. Moreover, cells in ThromboSol-treated PCs (platelets, polymorphonuclear cells, and monocytes) may be inhibited by the second messenger inhibitors of ThromboSol and thus, their capacity to bind and clear activated complement fragments may be impaired compared to that of cells present in control PCs.

In summary, our study confirms and extends previous work showing that refrigerated storage reduces the accumulation of bioreactive substances (IL-6, IL-8, TGF-β1, C3a and C4a) in the PCs. At present, the precise role of these compounds as mediators of FNHTR or other adverse reactions, and the dose required to trigger such reactions, are still uncertain.[36] Therefore, the clinical benefits of reduced levels of these substances in PCs are speculative. Likely, some patients, such as those with recurrent FNHTRs, would benefit from receiving PCs containing low levels of bioactive compounds, but only randomized and prospective clinical trials will give a satisfactory demonstration of whether this is, indeed, true. In this study, ThromboSol, an agent designed to sustain storage of PCs at 4ºC by protecting platelets from cold damage, does not interfere with the cold-promoted reduction in the accumulation of IL-6, IL-8 and TGF-β1. Indeed, by itself, ThromboSol, independently of the temperature, promotes lower levels of leukocyte-cytokines (IL-6 and IL-8) in PCs, but seems to have a detrimental effect on the storage-promoted accumulation of anaphylatoxins. The refrigeration-ThromboSol system appears to be a potential alternative to current platelet storage conditions, possibly allowing longer shelf-life of PCs without increasing side-effects in recipients. Extending the shelf-life of stored platelets would help to ameliorate the current problems of PC outdating and inventory management in blood banking.

Contributions and Acknowledgments

JR and VV designed the study. MLL, JR and JC were involved in the preparation, storage, and sampling of PCs. FF carried out the measurements of bioactive compounds in PC samples, and performed the statistical analysis of data. JR and FF wrote the manuscript, which was revised and approved by all other authors.

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Disclosures

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Potential implications for clinical practice

Currently approved PCs have important drawbacks such as a short shelf-life and a time-dependent accumulation of products potentially causing FNHTRs in recipients. This study deepens the investigation into the potential benefits of refrigerated storage of PCs supplemented with ThromboSol. We show that the application of these conditions may an extend the storage of PCs, while impairing the accumulation of compounds potentially harmful to recipients. The development of safe and effective novel platelet products, with extended shelf-life, would help to ameliorate the current problems of PC outdating and inventory management in blood banking.

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