Effect of Sialic Acid on Platelet Cryopreservation

G. Hayri ÖZSAN, Özden PİŞKİN, Fatih DEMİRKAN, Halil ATEŞ,
Mehmet A. ÖZCAN, Bülent ÜNDAR

Department of Hematology-Oncology, School of Medicine, University of Dokuz Eylül, İzmir, TURKEY

ABSTRACT

Sialic acid is a molecule which is responsible for the net negative surface charge of platelets. We investigated the effect of sialic acid on fresh and cryopreserved platelets. Platelet samples were obtained by platelet apheresis from 8 healthy donors. Platelet suspensions with different sialic acid concentrations (0, 1, 2 and 4 mg/mL) were studied for ADP and ristocetin induced platelet aggregation, basal and ADP induced P-selectin and glycoprotein-Ib/IX expression. Then platelet samples were cryopreserved in 5% DMSO with or without 4 mg/mL sialic acid. After thawing, P-selectin expression was compared with the control group. Six samples were also washed after thawing and P-selectin expression was again compared to unwashed samples.

Sialic acid suppressed ADP induced platelet aggregation and P-selectin expression in a dose dependent manner. In cryopreserved samples, P-selectin expression of 4 mg/mL sialic acid containing group was found significantly higher than the control group ($p< 0.001$). In cryopreserved control group, P-selectin expression of thawed-washed group was significantly higher than thawed-unwashed group ($p< 0.05$).

Our results indicate that sialic acid is not a good cryoprotective agent. Washing procedure after thawing to eliminate DMSO causes significant platelet activation.

Key Words: Platelets, Sialic acid, Cryopreservation, DMSO.

Turk J Haematol 2001;18(4):251-257.

Received: *21.12.2000* ***Accepted:*** *08.06.2001*

INTRODUCTION

Alloimmunization is a serious problem in patients who require frequent platelet transfusions[1]. This problem led trials of autologous platelet conservation including cryopreservation. However, cryopreservation has some undesirable effects such as platelet activation and loss of viability[2-7]. Loss of sialic acid in platelets has been reported after recovery of cryopreserved platelets in previous studies[8,9].

Sialic acid (N-acetyl neurominic acid) which is lo-

cated on the cell membrane has a major responsibility in maintaining negative surface charge of platelets[10,11]. This negative charge prevents aggregation of platelets to each other and adhesion of platelets to endothelial cells. It has been reported that some positively charged molecules such as polylysin, dextran and cathionized ferritin can induce platelet aggregation[12]. On the other hand platelet aggregation induced by polylysin and dextran was inhibited by negatively charged polymers such as heparin or mucopolysaccharides[13]. In this study we investigated the effect of sialic acid on platelet aggregation and activation as well as its effect as a cryoprotectant in platelet cryopreservation.

MATERIALS and METHODS

Study Design

Study 1: Investigation of sialic acid in platelet aggregation and activation: Platelet suspensions containing 0 mg/mL (control), 1 mg/mL (approximate physiologic plasma concentration), 2 mg/mL and 4 mg/mL of sialic acid were prepared. All samples were incubated for 30 minutes at room temperature.

Platelet aggregation with adenosine-diphosphate (ADP) and ristocetin was studied using a platelet aggregometer. Glycoprotein Ib/IX (GP Ib/IX) expression and platelet activation (P-selectin expression) were studied by flow cytometry (FCM) with or without ADP induction.

Study 2: Investigation of cryoprotective effect of sialic acid: Two groups, one without sialic acid (control group) and the other containing 4 mg/mL sialic acid (sialic acid group) was established. All samples were incubated at room temperature for 30 minutes. Basal P-selectin expression was analysed by FCM. Subsequently all samples were cryopreserved. Following thawing procedure all samples were analysed again for P-selectin expression. pH measurement was done by blood gas analyzer in all samples (Nova Stat Profile M, Nova Biomedical, St. Waltham, USA). Six samples from each control and sialic acid groups were washed after thawing to eliminate DMSO and resuspended with autologous plasma. In all samples P-selectin analysis was repeated.

Methods

Samples: Platelet concentrates and autologous plasma were collected by apheresis (Cobe-Spectra, Lakewood, CO, USA) from 8 healthy donors who did not ingest any drug known to interfere with platelet functions for the previous 10 days. All donors were directed donors for patients who required platelet transfusions and for this purpose their informed consent were also obtained. All thrombopheresis samples were diluted with autologous plasma with a ratio of 1:1.

ADP and Ristocetin-induced Platelet Aggregation

Platelet samples were assessed for aggregation, with ADP (2 x 10^{-5} M) or ristocetin (1.5 mg/mL) on a whole blood aggregometer (560VS, Chrono-Log, Havertown, PA, USA). The aggregation was determined as the pecentage of maximum aggregation using platelet-rich plasma as the baseline and platelet-poor plasma as 100 percent.

Preparation of ADP induced platelets: After addition of ADP (2 x 10^{-5} M) to platelet suspension, platelet samples were incubated for 20 minutes at room temperature.

Cryopreservation procedure: All samples were cryopreserved in cryotube vials (Nunc cryotube vials, Nalge Nunc International Denmark) up to -100°C by controlled rate freezing in 5% DMSO final concentration. Then all vials were transferred to -196°C liquid nitrogen tanks and stored for 24 hours.

Thawing and washing procedure: After cryopreservation, samples were thawed in a water bath at 37°C in no longer than 5 minutes. Thawed samples were diluted in 10 fold PBS containing 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemark, Israel) and 10% CPDA-1 solution and centrifuged at 2500 rpm for 12 minutes at room temperature. After removing the supernatants platelets were resuspended with autologous plasma.

Flow-cytometric Analysis

Platelet samples were fixed in 1% para-formaldehyde (Merck KGaA, Darmstadt, Germany) for 2 hours at room temperature. Samples were stored at 4°C in dark for no longer than 12 hours.

Platelets were incubated with anti CD42b (CD42b FITC anti-human, Pharmingen, S. Diego, CA, USA) and anti-CD62P (CD62 PE, Becton Dickinson, San Jose, CA, USA) monoclonal antibodies for GP Ib/IX and

P-selectin expression, respectively for 30 minutes. Isotypic controls of both monoclonal antibodies were used. An argon-ion laser equipped FACScan flow-cytometry operating at 15 mW and 488 nM wave length was used for analysis (Becton Dickinson, FACS Calibur, S Jose, CA, USA). After gating platelets on forward angle light scatter (FS) and right angle side scatter (SS) plot, 10.000 events were counted. Data were obtained in logarithmic amplification mode. The fraction of CD42b or CD62P positive platelets in a sample was expressed in terms of the % of florescence signals that were larger than the upper boundary of non-specific immune fluorescence determined by negative isotypic control.

Statistics: All data were reported as mean ± standard error of mean (SEM). Paired-t test was used to determine differences in means between groups. $p< 0.05$ was considered significant.

RESULTS

Effect of Sialic Acid in Platelet Aggregation and Activation

ADP-induced aggregation response in platelet concentrates was decreased in parallel to increasing concentrations of sialic acid. ADP-induced aggregation was significantly suppressed in the sialic acid concentration of 4 mg/mL in comparison to the control (61.1 ± 7.3 vs 4.5 ± 2.2 and $p< 0.001$). No significant changes were observed in ristocetin response to increasing concentrations of sialic acid. (Figure 1).

In control group basal GP Ib/IX and P-selectin expression was 98.6% ± 0.3 and 3.1% ± 1.6, respectively. Increased concentrations of sialic acid did not cause a significant change in Gp Ib/IX and P-selectin expression. However in ADP-induced samples, P-selectin expression was depressed in parallel to increasing concentrations of sialic acid (Figure 1 and Figure 2). This depression was statistically significant when 4 mg/mL sialic acid containing samples were compared to control group: 15.5% ± 5.0 vs 39.1% ± 5.3 ($p< 0.01$).

Investigation of Cryoprotective Effect of Sialic Acid

In control group, P-selectin expression did not show any significant change before and after cryopreservation (3.83 ± 1.55 vs 5.57 ± 1.13). In sialic acid group P-selectin expression was significantly higher in the post-cryopreservation samples compared to pre-cryopreservation samples (26.36 ± 3.25 vs 1.28 ± 0.34) ($p< 0.001$). Post-cryopreservation P-selectin expression in sialic acid group was significantly higher than the control group ($p< 0.001$). Post cryopreservation pH levels were significantly lower than post cryopreservation control group (7.02 ± 0.11 vs 7.59 ± 0.96 and $p< 0.05$).

In control group P-selectin expression of post- thawing washed samples (24.87 ± 4.73) was higher in comparison to unwashed samples (6.4 ± 1.26, n= 6 and $p< 0.05$) (Figure 3). In sialic acid group, post-thawing washing procedure did not change P-selectin expression significantly.

DISCUSSION

In patients who require frequent platelet transfusions, platelet alloimmunization may be a serious and life threatening problem[1]. Although HLA-and HPA-compatible platelet transfusions may solve this problem partially they require well organization. On the other hand although conservation and autologous transfusion of platelets seem to the best solution in theory, the main issue is that an optimal platelet storage procedure has not yet been defined. Due to their short life spans, platelets can be stored for a long time by only cryopreservation[14]. However, cryopreservation may cause undesired effects on platelets like decreased aggregation response, decreased resistance to hypotonic stress, loss of dense granule pool and changes in morphology, mitochondrial damage[2-7].

Sialic acid which is mainly responsible for the negative charge of platelets may be lost due to cold exposure[8,9]. In the first part of our study, we investigated the effect of high concentrations of sialic acid on platelet aggregation and activation. In a dose dependent manner sialic acid inhibited ADP-induced platelet aggregation. At 4 mg/mL concentration that inhibition was almost complete. Similar to our findings, Costello et al showed that alpha-1 acid glycoprotein which contains a remarkable amount of sialic acid, inhibited ADP and adrenaline-induced platelet aggregation[15]. However we couldn't be able to demonstrate the same effect with ristocetin-induced aggregation. Previous studies indicate that positive charged molecules may enhance whereas negative charged molecules may inhibit ADP-induced aggregation[12,13]. It is possible that platelets absorb the sialic acid which increases the negative sur-

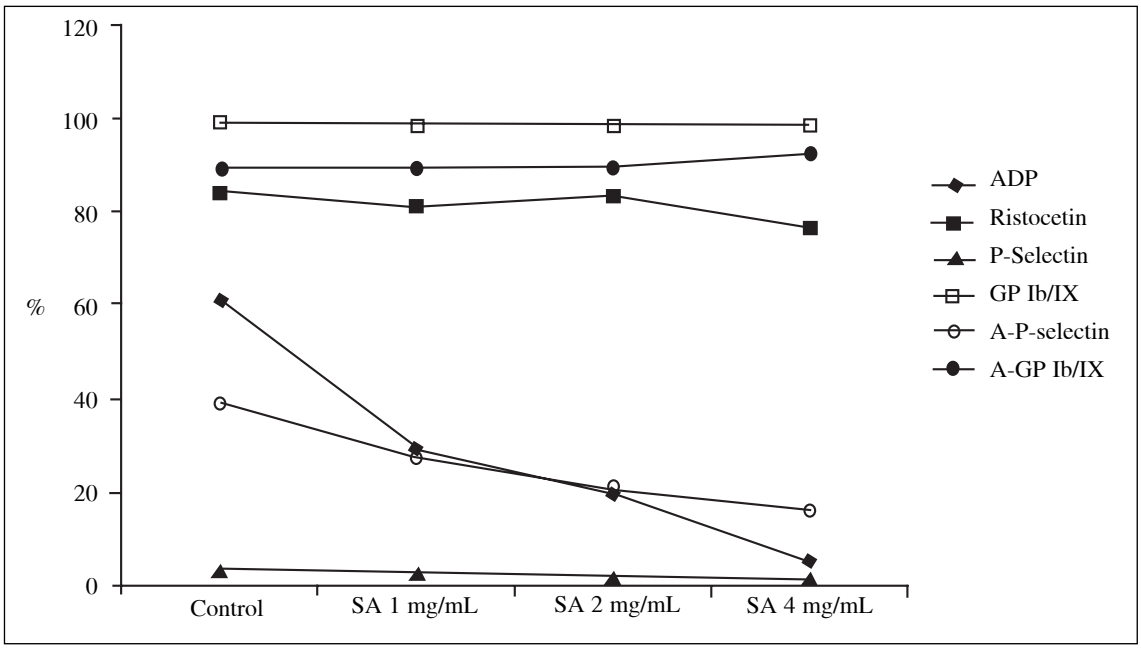

Figure 1. Effect of different concentrations of sialic acid (SA) on, P-selectin and glycoprotein Ib expression, ADP and ristosetin-induced platelet aggregation, ADP-induced P-selectin (A-P-selectin) and glycoprotein Ib (A-GP Ib/IX) expression.

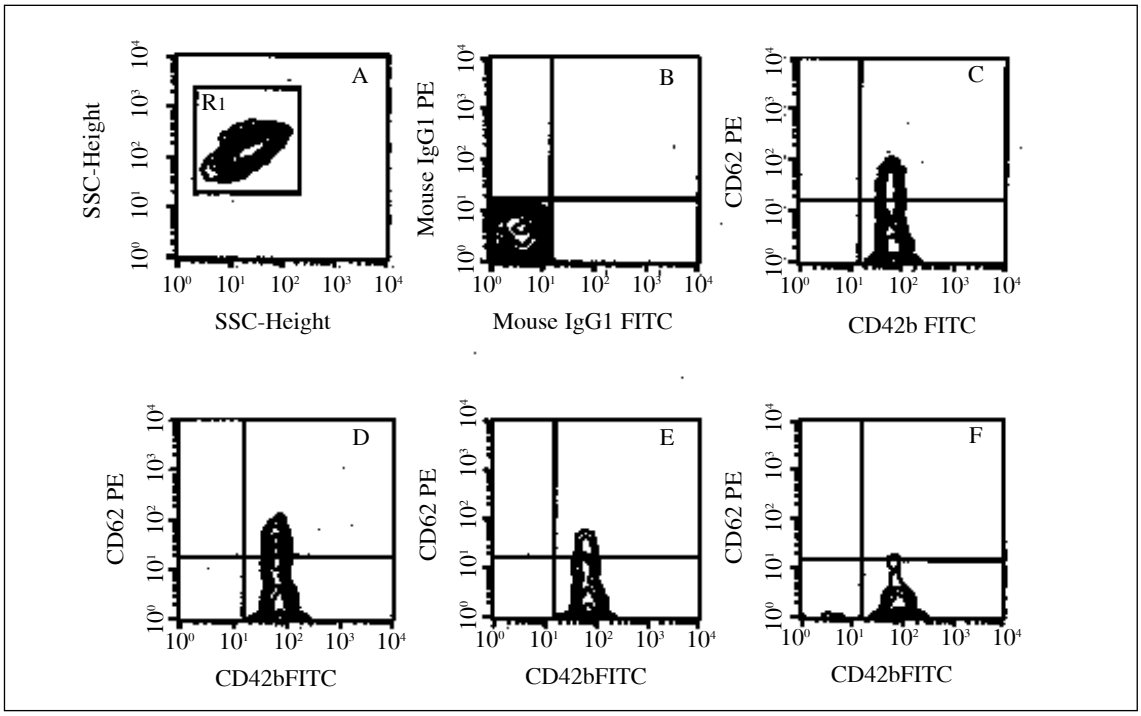

Figure 2. Flow-cytometric analysis of P-selectin (CD 62) and glycoprotein Ib/IX (CD42b) expression in different sialic acid concentrations (A: Gated platelets, B: Negative isotypic control, C: Control, D: Sialic acid 1 mg/mL, E: Sialic acid 2 mg/mL, F: Sialic acid 4 mg/mL).

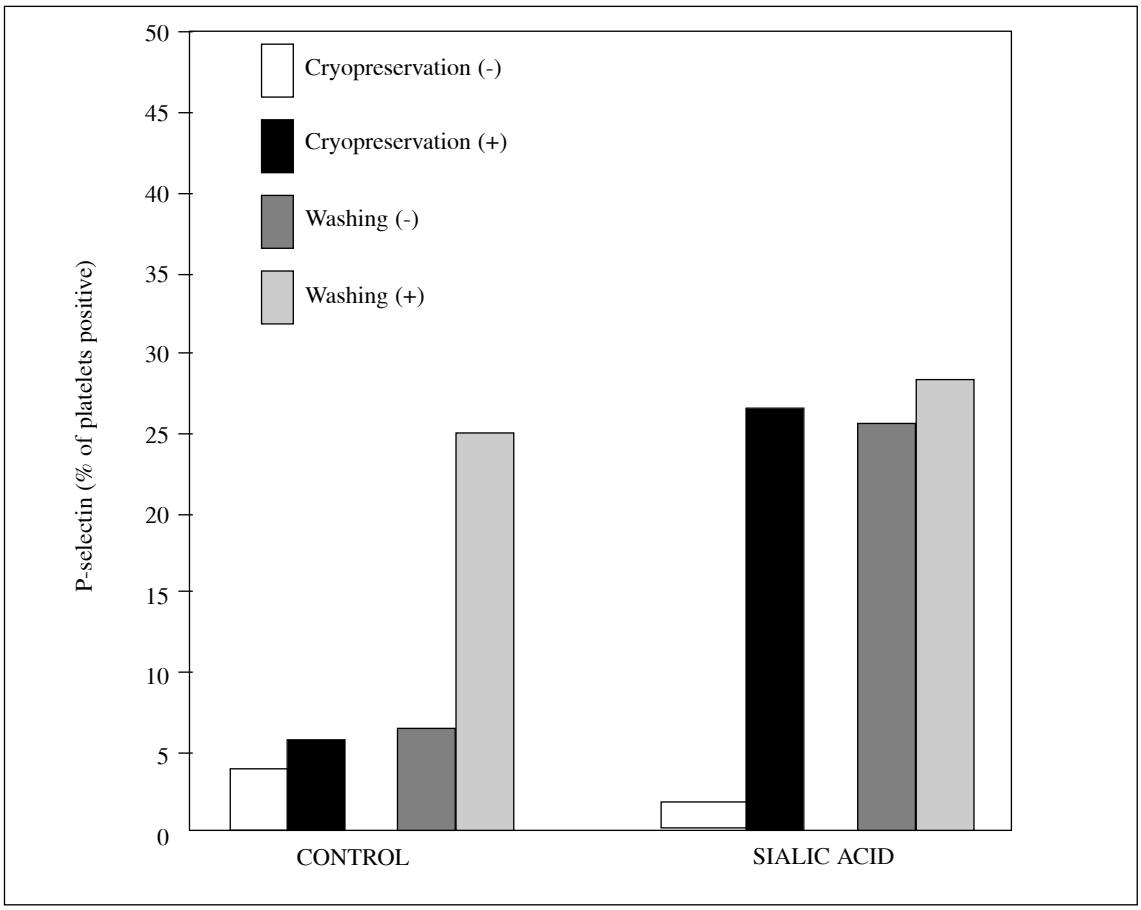

Figure 3. Effect of cryopreservation (n= 8) and washing (n= 6) on platelet P-selectin expression in control and sialic acid (4 mg/mL) groups.

face charge and decreases their contact to each other. In ADP-induced aggregation platelet-platelet contact is an important factor[16]. On the other hand, in our study, ADP induced P-selectin expression was conversely proportional to the sialic acid concentration. Our findings indicate that sialic acid inhibits ADP-induced P-selectin expression which shows platelet activation[17]. The absence of a significant change in GP Ib/IX expression excludes the antigen masking feature of sialic acid.

An ideal cryoprotective agent in platelet cryopreservation should inhibit platelet activation during the procedure and storage but this effect should disappear completely after re-infusion. In the second part of our study, we investigated the cryoprotective effect of sialic acid depending on our results showing that 4 mg/mL sialic acid concentration may be an effective inhibitor of platelet aggregation and activation. Surprisingly, after cryopreservation and thawing procedure, in the group which we added 4 mg/mL sialic acid in addition to 5% DMSO, we observed a significant rise in P-selectin expression. In the sialic acid group the pH levels of samples were significantly lower than the control group. It is well known that at pH levels below 6.7 reversible changes begin in platelets and pH levels below 6.1 are associated with irreversible loss of viability[14]. However our mean pH value was 7.02 in sialic acid group. For this reason we speculate that DMSO and/or cryopreservation may cause some conformational or structural changes in the platelet membrane in the presence of excessive amounts of sialic acid in the environment.

A second result of our study was that washing procedure in cryopreserved/thawed platelets increased

platelet activation significantly. As in stem cell transplantation products containing DMSO has been transfused for years with tolerable side effects it might be advisible to use cryopreserved platelet suspensions without washing[18].

Our data show that sialic acid can not be proposed as a cryoprotective agent for platelets. Optimal conditions and new cryoprotective agents still need to be investigated in platelet cryopreservation.

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Address for Correspondence:

G. Hayri ÖZSAN, MD

Department of Hematology-Oncology
School of Medicine, University of Dokuz Eylül
35340, İnciraltı, İzmir, TURKEY