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The effects of platelet apheresis in total hip replacement surgery on platelet activation

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Background: Autologous platelet rich plasma (PRP) harvest with autotransfusion devices has been used for 10 years in cardiac surgery and recently in orthopedics as a blood saving method. The quality of the harvested platelets has not been adequately examined, in part because of methodological difficulties in studying platelet function during surgery.
Methods: Twenty patients undergoing primary total hip replacement (THR) were studied. Ten patients underwent an immediate preoperative platelet apheresis to obtain concentrated platelet rich plasma (c-PRP). The other 10 patients not undergoing apheresis were allocated to a control group.
Platelet activation was evaluated as the population expressing P-selectin on the surface of platelets in the c-PRP and in blood samples collected pre-, per- and postoperatively. The method used was flow cytometry.
Results and conclusions: A minor population of activated platelets was found to be circulating in the patients' blood, with a highly significant difference between patients ($P = 0.005$), and with a range of 1–23% in peroperative activation. PRP harvest did not significantly alter platelet activity.
The platelet apheresis procedure did not inhibit platelet function in the c-PRP, as judged by a high proportion of platelets that could be activated in ADP stimulation experiments (mean value ± SD 86% ± 7.5%).

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Key words: ADP; autotransfusion; cytometry; flow P-selectin; platelet; plateletpheresis.

PREOPERATIVE platelet rich plasma (PRP) harvested with autotransfusion devices, regularly used for intraoperative autotransfusion (IAT) and postoperative blood drainage sampling, has been used for 10 years in cardiac surgery with varying results (1–5) with respect to bleeding and allogeneic blood transfusions. In liver and spine surgery (6–8) PRP has been used to counteract the intraoperative coagulation defects caused by dilution related to cell washing with saline during IAT, hemodilution and the use of different intraoperative plasma substitutes.

In a previous study, we showed PRP harvest in combination with autotransfusion to be as effective in reducing allogeneic blood usage in orthopedic surgery as the combination of autotransfusion and predonation of two units of blood (9). Some studies have examined the effect of PRP on blood coagulation (3, 4) and on platelet aggregation (10), but to our knowledge no study has evaluated the quality and effect of the harvested platelets with a method that discriminates between resting and activated platelets. Flow cytometry, which is used here, is such a method. It is based on the detection of P-selectin (GMP 140), a protein of the a-granule membrane, which fuses with the plasma membrane during secretion when the platelet becomes activated (11, 12). With this technique we evaluated the platelets in pre-, per- and postoperative blood samples from all patients, and also in the collected concentrated platelet rich plasma (c-PRP) suspension just before reinfusion.

The purpose of this study was to evaluate:

1 if there are any differences between groups or between individuals in the population of platelets expressing P-selectin (as an activation marker) in samples collected from patients perioperatively.
2 if c-PRP platelets are activated by the platelet activator adenosine diphosphate (ADP) in the same manner as platelets in preoperative blood samples.

Materials and methods

Twenty patients, mean age 67 years, with severe hip arthrosis undergoing primary total hip replacement (THR) were investigated in a prospective, consecutive, non-randomized study. The non-randomized de-

sign was chosen because of logistics problems (availability of personnel, apparatus and patients). Only patients with ASA I-II, no history of platelet disorder or abnormal bleeding, normal platelet counts and without anticoagulative medication were accepted. The local hospital ethics committee approved the study. Informed consent was obtained from each patient.

Platelet inhibiting drugs were withdrawn 10 days preoperatively. A daily dose of 5000 IU of low molecular weight heparin (Fragmin®, Pharmacia, Sweden) was given subcutaneously the evening before surgery and every evening up to 10 days postoperatively, as this is our routine at the hospital. Ten patients underwent immediate preoperative PRP harvest (done before initiation of anesthesia) with an AT 1000 autotransfusion device. The aim was to collect 25–30% of the total amount of the patient's platelets, as this amount has been shown to reduce bleeding in cardiac surgery upon retransfusion (2). Blood was retrieved through a central venous catheter into a 225-ml bowl, and acid-citrate-dextrose (ACD, Baxter, IL) was used for anticoagulation. Separation of blood into erythrocytes, platelets and plasma was continued until 225 ml of erythrocytes were collected in the centrifuge bowl. First, a high initial centrifugation rate of 5600 r.p.m. was used, and platelet poor plasma was subsequently pooled with the erythrocytes and returned to the patient. Then a lower centrifugation rate of 2400 r.p.m. was applied to obtain PRP. This fraction of about 100 ml contained the buffy coat and the first 50 ml of erythrocytes to maximize the yield of platelets and was called concentrated PRP (c-PRP). Central venous pressure (CVP) and mean arterial pressure (MAP) were maintained with 1000 ml of 4% albumin. Blood was processed for up to five cycles, 12–15 min each. The platelet apheresis procedure was monitored by a platelet count in the c-PRP and by calculating the total platelet yield expressed as a percentage of the calculated total blood platelet count (2). The c-PRP was stored at room temperature, agitated intermittently and infused immediately after wound closure. Six of these patients were also participating in a study examining the efficiency of PRP harvesting as a blood component saving technique (9). Ten other patients were allocated to a control group and did not undergo platelet apheresis. IAT was performed with the autotransfusion device up to 2 h postoperatively in all patients except for two in the control group and one in the PRP group. Invasive arterial pressure (PRP group), hourly measurement of diuresis, a three-lead electrocardiogram and pulse oximetry were routine.

After premedication with intramuscular oxicodon-scopolamine and preloading with 500 ml of Ringer-acetate, a combined lumbar spinal-epidural anesthesia (CSE, needle through needle) was administered with 4 ml of 0.5% bupivacaine (Astra, Sweden). The patients were operated upon in a horizontal lateral position. Vacuum mixed cement was injected with a syringe in a retrograde direction. The proximal femur was sealed and additional cement was injected under pressure. The femoral prosthesis was inserted during the viscous phase of the cement. Cloxacillin 2 g was given prophylactically during the operation.

To estimate the activity of the platelets, arterial blood samples were drawn prior to the PRP harvest or induction of anesthesia (control group), 15 min after the start of surgery, and immediately postoperatively. On each occasion 4.5 ml of blood was drawn into a siliconized tube containing 0.5 ml of buffered trisodium citrate 0.129 mol/l (Vacutainer Systems, Becton Dickinson AB, CA), allowing for the detection of platelets that could be *in vitro* activated with ADP. A second 4.5 ml sample of blood was collected into a siliconized tube containing CTAD inhibition solution (0.5 ml of trisodium citrate 0.11 mol/l, theophylline 15 mmol/l, adenosine 3.7 mmol/l and dipyridamole 0.198 mmol/l, pH 5.0), and a third sample of 3 ml of blood was collected into a tube containing 0.072 ml of K_3EDTA 0.17 mol/l for inhibition. The latter was a reserve sample for those cases in which the CTAD solution was less effective.

The tubes containing the blood samples were gently swayed on a flat bed shaker for 30 min and then analyzed within 3 h. In accordance with the following procedure, 10 µl of whole blood in buffered trisodium citrate 0.129 mol/l was added to round-bottom 12 × 75 mm polypropylene tubes (Falcon®, Becton-Dickinson AB) containing either 50 µl of HEPES buffer, pH 7.40 (NaCl 137 mmol/l, KCl 2.7 mmol/l, $MgCl_2$ 1 mmol/l, glucose 5.6 mmol/l, bovine serum albumin 1 g/l, and HEPES 20 mmol/l, pH 7.40 (13). When studying *in vitro* platelet activation, 50 µl of HEPES buffer pH 7.40 containing ADP was added to obtain a final concentration of 100 µmol/l. In order to avoid postcollection *in vitro* platelet activation, 10 µl of whole blood collected in CTAD mixture or in EDTA was added to two separate tubes containing 50 µl of HEPES buffer pH 7.40 and EDTA 100 mmol/l. After gentle mixing, the samples were stored at room temperature without stirring for 10 min 10 µl of phycoerythrin-labeled anti-P-selectin (CD 62P) (Becton and Dickinson AB) was added and the tubes were incubated for an additional 20 min, followed by the addition of 500 µl of isotonic buffered 1% formaldehyde solution, CELLfix (Becton Dickinson AB).

Flow cytometry was performed using a FACScan

flow cytometer with Argon laser (Becton-Dickinson AB). The software LYSISII® (Becton and Dickinson AB) was used for both acquisition and analysis of data from the cytometer. The forward scatter (FSC), side scatter (SSC), and fluorescence channel 2 (Fl-2) detectors were logarithmically amplified. The live gate around the platelet population was identified by its light scatter characteristics using a FSC/SSC dot plot. 10000 live gated events were acquired and stored on the computer for further analysis. The FL-2-height overlay histogram from the preoperative sample that was collected in a CTAD tube and ADP-stimulated was used to determine the cross point discriminating platelets expressing P-selectin as a marker for activation. Platelets below this point were considered to be inactivated. This procedure was applied for each patient, and the same discriminating point was used for all statistics for that particular patient (*Fig. 1*). The percentage of activated platelet events was calculated in relation to the 10000 events set as 100%.

Statistical analyses

The individual changes from the starting values for spontaneous and ADP-mediated activation were analyzed by a three way ANOVA (analysis of variance), with the factors time, treatment and patient within treatment. The first two factors were considered as fixed factors and the last (patient within treatment) as a random factor. Further, the differences between per- and postoperative data for spontaneous and ADP-mediated activation versus preoperative data were tested by ordinary *t*-tests with Bonferoni correction (significance level 0.05/2). In the absence of a significant treatment effect, the data was analyzed as a randomized block design with the factors patient and time. For the PRP patients a paired *t*-test was done to compare the ADP-mediated activation in preoperative blood samples with ADP-mediated activation in the PRP solutions. Finally, the patient effect was studied on the original data both for spontaneous and ADP-mediated activation with a two-way ANOVA with the factors time and patient.

Results

We used the cross point between CTAD-inactivated and ADP-activated platelets in the preoperative blood sample to discriminate resting and activated platelets in all samples from the same patient (*Fig. 1*). The proportion of the platelet fraction not expressing P-selectin varied between 90 and 99% (mean 96%, SD 2.3%) preoperatively for the 20 patients as a group (*Table 1*). The differences between the proportions of spontaneously activated platelets during the different

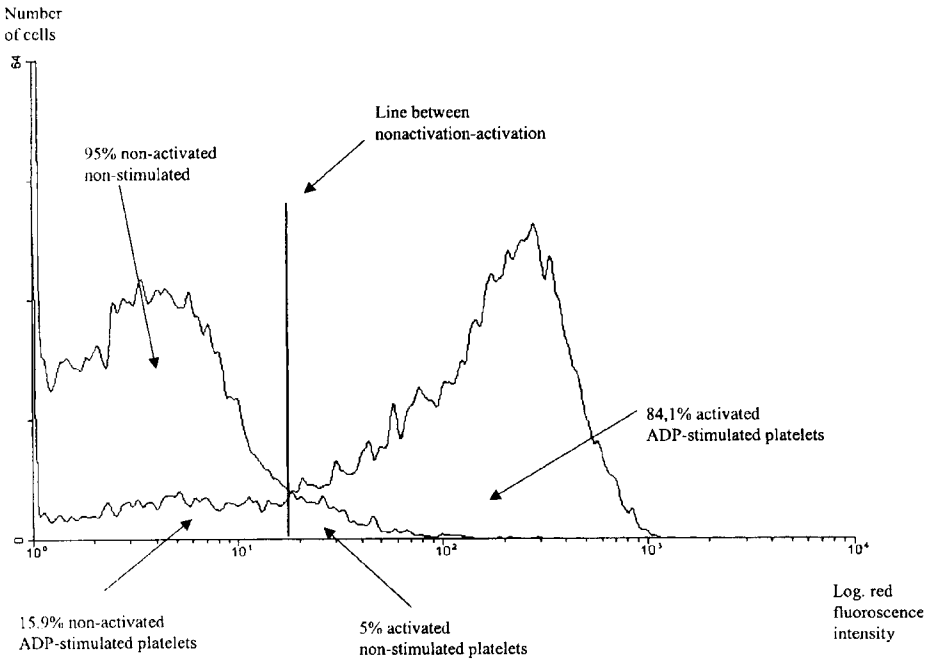

Fig. 1. Preoperative flow cytometry picture of patient no. 19 showing the differentiation between spontaneous and ADP induced P-selectin expression on the platelet surface. The curve to the left contains CTAD-inactivated platelets and the curve to the right ADP-stimulated platelets. The platelets in the area on the left side of the cross point are referred to as nonactivated and the platelets on the right side of the cross point are referred to as activated, i.e. expressing P-selectin.

phases of the operation were interpreted as being mainly the result of the patient's treatment, since the gating to distinguish between activated and-non-activated platelets was the same and unique for each patient. There were no significant differences in height or weight among the patients. The patients in the PRP group were significantly younger ($P<0.05$) than the patients in the control group (63 years and 70 years, respectively).

The individual data for each patient and the means ± SD for each group for pre-, per- and postoperative platelet activation are shown in Table 1. There was no treatment effect, i.e. there were no significant differences in platelet activation, either spontaneous or ADP mediated, between the PRP group and the control group. There was a significant time difference between the pre- and peroperative periods ($P<0.002$) and ($P=0.00005$) for spontaneously activated and ADP activated platelets, respectively. For ADP activated platelets there was also a significant time difference ($P=0.015$) between the per- and postoperative periods. There were highly significant differences in the patients' expression of P-selectin during the pre-, per- and postoperative periods, $P=0.005$ for the spontaneously activated platelets and $P<0.0001$ for the ADP activated platelets, i.e. there was great variability within each group.

The spontaneous and ADP mediated platelet activation in all c-PRP solution bags as well as means ± SD are shown in Table 2. In the patients undergoing PRP harvesting, the c-PRP solution contained between 25 and 45% of the patient's blood platelets (*Table 2*). The platelets in the c-PRP suspension showed a high degree of spontaneous activation with values up to 31% (*Table 2*). The ADP stimulated activation was, however, not impaired. Thus, in the c-PRP from seven of the 10 patients the proportion (%) of platelets activated by ADP exceeded (not significantly) that in the preoperatively obtained blood sample (*Tables 1 and 2*).

Discussion

Platelets play an essential role in hemostasis, and their state of activity can be altered during surgery. A platelet saving technique – PRP harvest with autotransfusion devices – has been shown to reduce postoperative blood loss in cardiopulmonary surgery (CPB) (1,

Table 1

Patient data: bleeding and platelet activation

PRP Patient no:	Preoperative activation (%)	Peroperative activation (%)	Postoperative activation (%)	ADP-stimulated activation (%)	ADP-stimulated activation (%)	ADP-stimulated activation (%)
1	6	2	4	86	75	83
2	4	12	0	82	78	X
3	8	23	20	79	69	75
4	5	16	4	88	X	86
5	7	8	12	83	X	72
6	1	20	3	80	74	75
7	4	4	4	91	86	91
8	3	4	8	81	83	82
9	3	5	5	81	79	86
10	3	4	6	84	81	88
rnean ± SD	4.4 ± 2.1	9.8 ± 7.5	6.6 ± 5.7	83 ± 3.8	78 ± 5.4	82 ± 6.6
Control Patient no:						
11	3	3	3	88	89	88
12	5	22	2	60	X	71
13	10	X	13	87	X	75
14	2	2	2	92	86	89
15	2	2	1	89	87	83
16	5	8	6	79	77	85
17	2	4	3	91	87	88
18	3	1	2	81	75	59
19	5	2	5	84	77	85
20	2	2	2	91	87	91
mean ± SD	3.9 ± 2.5	5.1 ± 6.7	3.9 ± 3.5	84 ± 9.6	83 ± 5.7	81 ± 10

Data for each individual, numbered patient showing the percentage of the patient's platelets expressing the activation marker P-selectin either spontaneously or after activation with ADP.
PRP = platelet rich plasma group, Control = control group, x = missing value.

Table 2

The c-PRP concentrate platelet yields and response to activation with and without ADP

Patient	Platelet yield (%)	Spontaneously activated (%)	Activated by ADP (%)
1	28	7	83
2	28	26	88
3	32	22	77
4	36	18	93
5	38	28	87
6	45	9	70
7	X	22	94
8	26	19	89
9	26	16	86
10	25	31	92
mean ± SD	31.6 ± 7.2	19.8 ± 7.7	85.9 ± 7.5

Data for each individual, numbered patient showing the percentage of platelets in the c-PRP expressing activation marker P-selektin either spontaneously or after activation with ADP.
x = missing value.
Platelet yield was calculated as the relationship between the total number of platelets in the c-PRP concentrate and in the circulating blood.

2) and orthopedic surgery (9). However, not all studies have shown blood saving effects (3, 4). The single most important factor seems to be the percentage of platelets that are harvested, and it is necessary to harvest a minimum of 20% of the patient's platelets in order to have efficient blood loss reduction (1). Whereas the effects of plasmapheresis on platelet aggregation have been studied (10), to our knowledge there is no previous study dealing with activation at the time of sampling and further potential activation of the preoperatively harvested platelets. The platelets in the c-PRP solution should be fully effective in hemostasis during the postoperative period due to the short storage interval (up to 4 h). Can the apheresis and storage affect the platelets, thereby making them less effective?

P-selectin is an integral membrane glycoprotein found in a-granulae of platelets (11, 12). After platelet activation it is rapidly redistributed to the plasma membrane. We assessed platelet activation by using flow cytometry to measure platelet surface expression of P-selectin (13). Gutensohn et al. (14) examined the effect of continuous flow platelet apheresis (as used in blood centers) on the donor's blood and found platelet activation with expression of P-selectin in all 22 donors. For 57% of the donors this returned to normal within 24 h. Wun et al. (15) found that up to 87% of the platelets expressed P-selectin in samples collected from plasma donors after plasmapheresis in a blood center. Although collection could induce some activation, a massive influence on the platelets caused by the apheresis process seems probable, but has not been studied adequately in the apheresis procedure with autotransfusion devices used in the operating room. In our study, we could not demonstrate that the apheresis process had any effect on spontaneous platelet activation in the patient's blood, whereas surgery in itself did have such an effect. We therefore believe that PRP harvest does not induce any platelet-mediated hypercoagulation and can safely be used during THR surgery. However, surgery did lead to increased platelet activation, corroborating the results from studies on coronary surgery (16, 17).

Studies on platelet concentrates have demonstrated a progressively increasing activation of the platelets during the storage period (18–20). It has been shown that in platelet concentrates harvested as PRP, up to 20% of the platelets initially expressed P-selectin, at a maximum of 60%, after 9 days (20). However, it is difficult to compare results from different investigations, as there are great variations in reported P-selectin expression, even when the same sample is analyzed (21). In our study we found a range of 7–31% of the platelets expressing P-selectin in our 10 c-PRP concentrates. We wanted to examine the percentage of platelets that could be activated when stimulated, indicating a capacity to react to coagulative stimuli. This was tested *in vitro* by activating the platelets with the physiological activator ADP. In seven of the 10 PRP patients, the fraction of platelets in the c-PRPs that could be activated by ADP was higher than in blood sampled preoperatively, indicating that the apheresis process did not damage the c-PRP platelets.

In conclusion, a minor population of platelets presenting P-selectin (as a marker of activation) was found to be circulating in the patients' blood, with highly significant differences between patients ($P = 0.005$). However, PRP harvest did not significantly alter platelet activity, whereas platelet activation was significantly higher peroperatively compared to preoperatively.

The platelets in the c-PRP are not inactivated. They can be activated *in vitro* by physiological activators like ADP in the same manner as platelets are activated in the blood preoperatively.

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