Haematologica 2000; 85:381-385
original paper

Hemostasis & Thrombosis

Platelet function during cardiopulmonary bypass not changed by two different doses of aprotinin

Amparo Santamaría, Jose Mateo, Eduardo Muñíz-Díaz,# Arturo Oliver,* Joaquín Murillo, Héctor Litvan,° Juan C. Souto, Jordi Fontcuberta

*Department of Thrombosis and Hemostasis, *Puigvert Fundation, #Blood Bank and °Anesthesiology, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain*

Abstract

Background and Objectives. **Bleeding is one of the major complications of cardiopulmonary bypass (CBP) during cardiac surgery. A platelet function defect seems to be the main cause of the hemostatic problems associated with CBP. Controversial results have been reported concerning the possible protective mechanism of action of aprotinin on platelets.**

Design and Methods. **In this study we investigated the effect of two different dosages of aprotinin (high and pump-prime-only dose) on platelet reactivity *in vitro* and adhesion, activation and aggregation receptors on the platelet surface.**

Results. **The results obtained from 53 patients undergoing CBP showed a significantly deficient platelet aggregation in response to agonist in all groups without differences between aprotinin treated or not treated patients. No changes in platelet surface expression of glycoprotein (GP) IIb-IIIa, GPIb, GPIV and P-selectin, were observed during CBP between patients treated with aprotinin or not.**

Interpretation and Conclusions. **These data suggest that inadequate platelet function induced by CBP is not a defect intrinsic to the platelet. We conclude that the hemostatic effect of aprotinin, regardless of the dose employed, is not mediated by protection of platelet function.**

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Key words: aprotinin, cardiopulmonary bypass, platelets, glycoproteins, aggregations

Bleeding is one of the major complications of cardiopulmonary bypass (CPB) during cardiac surgery. Inadequate platelet function seems to be a main defect of hemostasis during CPB due to extensive contact of blood with synthetic, non-biologic surfaces which contributes to blood loss.[1-4] Other factors also contribute to this coagulation defect, such as the oxygenator, cardiotomy suction, hypothermia, drugs, hemodilution and duration of CPB.[2] Some studies have reported a platelet function defect during CPB. The nature of this alteration is still controversial, although it seems to be related to changes in quantitative or qualitative expresion of major platelet surface receptors such as von Willebrand receptor (glycoprotein [GP] Ib), fibrinogen receptor (GPIIb-IIIa), thrombospondin receptor GP(IV), and platelet activation-dependent granule-external membrane protein (P-selectin or GMP-140) and also a deficient platelet reactivity *in vitro*.[2,5,6] Kestin *et al.* have postulated that the platelet function defect of CPB is not intrinsic to the platelet, but is an extrinsic defect caused by, for example, the lack of availability of platelet agonist *in vivo*.[1] Bertolini *et al.* concluded that platelet composition and function may be affected very little or severely damaged during CPB for cardiac surgery probably depending on some aspects of the technical procedure.[7] Thus, the exact mechanism of the functional platelet defect during CBP is still unknown, and subsequently the effects of aprotinin on platelet function are poorly understood. Aprotinin (Trasylol®; Bayer, West Haven, CT, USA) is a non-specific proteinase inhibitor of plasmin, kallikrein, thrombin, trypsin, and chymotrypsin. It has been widely used in patients undergoing CPB during open heart surgery as standard treatment due to its proven efficacy in significantly decreasing post-operative blood loss and transfusion requirements.[8] The mechanisms of action of aprotinin are mainly related to inhibition of plasmin activity and probably of thrombin generation. It has also been suggested by several studies that aprotinin protects platelets against the initial effect of CPB,[9-25] although recently other authors have reported contradictory findings suggesting that aprotinin does not directly influence platelet function.[26-28] Only a few studies have compared the effect of different doses of aprotinin[9,15,24] on platelet composition and function, and have yielded opposite conclusions.

The rationale of this study was to demonstrate whether aprotinin has different effects when given as a pump-prime-only dose or at a high-dose since some reports have suggested an initial beneficial effect of a pump-prime-only dose to platelet function based on the theory that the main damage on platelet function is produced during the first pass of blood through the circuit within the first five minutes of CPB.[2,24]

In order to assess the mechanism of action of aprotinin on platelet aggregation, activation and adhesion, and platelet reactivity *in vitro*, we designed a prospective randomized double-blind study using two different aprotinin doses.

Design and Methods

Patients

After ethical committee approval and informed consent, a multicenter, prospective, randomized, double-

Correspondence: Jose Mateo, M.D., Department of Hematology, Hospital de Sant Pau, C/Sant Antoni Maria Claret 167, 08025 Barcelona, Spain. Phone: international +34-93-29199193 – Fax: international +34-93-2919192 – E-mail: jmateo@hsp.santpau.es

blind study was performed. Fifty-three patients (41 males, 12 females) undergoing coronary artery bypass grafting were enrolled to evaluate the effect of aprotinin on platelet function. To be eligible, the patient had to be older than 18 years. The following criteria for exclusion from entry to the study were used: history of previous cardiac operation, possible exposure to aprotinin in the past, allergy or clotting disorder, severe cardiac failure (ejection fraction < 30%), and impaired renal function (serum creatinine level >2 mg/dL). Demographic data are summarized in Table 1.

Aprotinin administration and cardiopulmonary bypass technique

Aprotinin (Trasylol®, Bayer AG, Leverkusen, Germany) was administered in saline solution without additives or preservatives. The patients were randomized into three groups depending on the dose of aprotinin.

Group P. The control group received a bolus of saline solution, saline in the priming solution and a continuous infusion of saline solution (n=14).

Group H. The high-dose group received a bolus of 2 $\times 10^6$ KIU of aprotinin, 2×10^6 KIU of aprotinin in the priming solution and continuous infusion of aprotinin at a rate of 0.5×10^6 KIU every hour during CPB (n=16).

Group L. The low-dose group received an initial bolus of saline solution, 2×10^6 KIU aprotinin in the priming solution, and continuous infusion of saline solution during CPB (n=23).

Anesthesia and CPB

Anesthetic, surgical and CPB procedures were carried out according to the institutional protocols of the Hospital de Sant Pau (Barcelona, Spain) and were similar in all three groups. Anesthesia was induced with flunitrazepam (0.02 mg/kg body weight) and fentanyl (10 to 20 mg/kg body weight). Pancuronium bromide (0.1 mg/kg body weight) was used for muscle relaxation. Anesthesia was maintained with successive doses of fentanyl. Isoflurane (0.5-1%) was added to the ventilation system when needed.

The CPB device was primed with Ringer's solution, polygeline, and mannitol. Extracorporeal circulation was instituted at 28°C with an output of 2.2 to 2.4 L/m^2 per minute. Perfusion pressure was maintained between 50 and 80 mmHg. A cardioplegic solution containing mannitol (8.9 g/L), dextrose (4.5 g/L), potassium (30 mmol/L), chloride (113 mmol/L), sodium (82 mmol/L), and bicarbonate (20 mmol/L) was injected every 20 minutes into the aortic root.

Table 1. Demographic data and data from CPB.

Characteristic	*Low-dose aprotinin*	*High-dose aprotinin*	*Placebo*	*p*
No. of patients	23	16	14	NS
Age (yr)	60 (42-78)	62 (43-75)	62 (47-76)	NS
Sex (M/F)	18/5	14/2	10/4	NS
Duration of CBP (min)	102±27	123± 40	106±26	NS

**Results are expressed as mean ± standard deviation. NS: not significant.*

Anticoagulation was achieved with heparin (300 IU/kg body weight) injected into the right atrium. Successive doses of heparin were given during CPB. Because aprotinin can prolong activated clotting time (Hemochron 401 device, International Technidyne Corp., Edison, NJ, USA), patients in whom the first activated clotting time measurement was greater than 750 seconds received heparin to maintain the activated clotting time between 750 and 950 seconds and in those patients in whom the first activated clotting time measurement was about 400 seconds, activated clotting time was maintained between 400 and 500 seconds. All groups received heparin in similar dosages. When surgical procedures were finished and CPB was stopped, heparin was neutralized by an infusion of 1.5 mg protamine sulfate/100 IU of heparin administered.

Blood sampling

Blood samples were taken immediately before the operation (T1), at the end of CPB but before protamine administration (T2), 1 hour after protamine neutralization (T3) and 18-24 hours after the operation (T4). Blood mixed with 0.129 mol/L sodium citrate in a 10:1 proportion was obtained for analysis. The samples were centrifuged within 30 minutes at 3,600 rpm at 4°C. Aliquots of the plasma were snap frozen and stored at –40°C until analysis. Aggregations were studied in samples obtained at T1 and T3. Platelet rich (PRP) and platelet poor plasma (PPP) were obtained by centrifuging the blood at 800 rpm for 10 min and at 3,500 rpm for 20 min, respectively, at room temperature and then tested immediately.

Laboratory assessment

Hemoglobin concentration and platelet counts in whole blood were determined by routine analysis with a Coulter counter (Coulter Electronics, Luton, England).

Platelet aggregation in response to 15 mg/mL ristocetin, 2 mg/mL collagen and 2 mmol/mL ADP were studied by Born's method as described elsewhere, respectively, in PRP (adjusted to 250×10^9 platelet/L with PPP) in a aggregometer (Aggregocorder PA-3210 Menarini Diagnostics) after (T1) and before CPB (T3). Results are expressed as percentage of aggregation at 5 minutes. Flow cytometry was performed on formaldehyde (1%) fixed platelets. All samples were incubated for 5 min at room temperature with FITC-labeled monoclonal antibodies anti-GPIIb-IIIa (CD41a, Jansen), anti-GPIb (CD42b, Jansen), anti-GMP-140 (CD62, Lander) and anti-GPIV (CD36, Lander) all from Immunotech (Marseille, France). Isotype controls which do not recognize target structures on platelets were obtained from the same manufacturer and employed as negative controls in the analysis of antigens with high expression. For the analysis of activation-dependent antigens, *in vitro* activated blood samples from healthy subjects were used as positive controls and compared to stabilized non-activated blood samples as negatives controls. Samples were diluted with 300 µL of PBS/BSA and analyzed in a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San José, CA, USA). Light scatter and fluorescence signals were acquired at logarithmic gain, and 10,000 platelets in each sample were analyzed. Other mea-

surements were: D-dimer (normal range: <500 ng/mL) (Asserachrom D-dimer, Boehringer-Mannheim, Mannheim, Germany) and prothrombin fragment F1+2 (normal range: 0.4-1.1 nmol/L) (Enzygnost F1+2 micro, Behring, Marburg, Germany).

Statistical analysis

Results are expressed as the mean and standard deviations and as the 95% confidence interval (CI). Percentages are expressed with their CI. All relevant differences are shown as the CI of these differences. A *p* value less than 0.05 was considered statistically significant. Age, sex and duration of operation and extracorporeal circulation were compared among groups by one-way analysis of variance. Primary efficacy analysis was done on the total blood loss during the first 24 hours after operation by an analysis of variance with one factor and CPB as the co-variable. An analysis of variance for repeated measures with one factor (treatment) was carried out to analyze the blood profiles at different times, and CPB duration was used as the co-variable. Polynomial contrasts were applied within different trends of each treatment. Bonferroni's correction was applied for multiple comparisons. The evolution of biological parameters throughout the CPB period was assessed by analysis of variances for repeated measures (treatment as the factor and duration of CPB as the co-variable). Appropriate contrasts were applied to evaluate profiles between treatments. If no influence of the analyzed factors was observed, a simple analysis of variance for repeated measures was used. D-dimer values were analyzed with the use of logarithmic transformation.

Results

There were no significant differences between the treatment groups in demographic characteristics such as sex, age and duration of CPB.

Median total blood loss was 903 mL (range 723-1,081) in group H, 1,243 mL (range 1,015-1,470) in group L, and 1,422 mL (range 1,089-1,756) in group P. Significant differences were observed between high-dose and low-dose groups vs the placebo group ($p<0.01$).

Effect on platelet reactivity in vitro

Results are summarized in Table 2.

Ristocetin-induced agglutination of platelets decreased significantly in all groups at the end of CBP ($p<0.0001$), although there were no statistical differences when comparing aprotinin groups (H and L) to the placebo group. The mean of this decrement at the end of the operation in all groups, from baseline values was –38% (95% CI 27-50) ($p<0.0001$).

Collagen-induced platelet aggregation slightly diminished in all groups ($p<0.014$) at the end of CBP. The mean value of this reduction [–28% (95% CI 1-17) with regard to baseline values] was statistically significant ($p<0.05$). There were no statistical differences between groups.

ADP-induced aggregation at 2 µmol/mL decreased significantly in all groups; mean value of the reduction at the end of CBP was –21% (95% CI 1-13) ($p<0.02$). No statistical differences existed between treatment groups.

Table 2. Effect on platelet reactivity *in vitro*.

	High- dose aprotinin	*Low-dose aprotinin*	*Placebo*	*p*
Ristocetin (T1) (%)	67±39	69±55	87±25	NS
Ristocetin (T3) (%)	30±37	29±22	49±30	
Collagen (T1) (%)	97±10	96±9	91±21	NS
Collagen (T3) (%)	94±14	74±36	87±26	
ADP2 (T1) (%)	93±13	93±13	92±14	NS
ADP2 (T3) (%)	89±18	85±26	92±17	

**Results are expressed as mean ± standard deviation. ADP: adenosine diphosphate.*

Effect on platelet surface complexes

CBP induced a significant increment in P-selectin expression on the surface of circulating platelets ($p<0.05$) at all times although the expression was slightly lower after protamine neutralization compared to at times T2 and T4. In the placebo group, P-selectin increased at the end of CPB, and decreased at 18-24 hours, without reaching the levels at the beginning of CPB. In the low-dose group, this increase was similar to that in the placebo group, although at 18-24 hours levels were higher than in the other groups. In the high-dose group, levels also showed a slight increase during and after 18-24 hours, but to a lesser extent than in the other groups. No statistical differences were observed between groups (Figure 1). The effect of CBP on the thrombospondin binding protein GPIV did not result in any significant change in the platelet surface expression of GPIV in the placebo group or aprotinin-treated groups. At the end of CPB before and after protamine neutralization there was a slight, but not statistically significant, increase in all groups. Surface expression of fibrinogen receptor showed no change during CBP in any group. Similarly surface expression of von Willebrand receptor did not change significantly in any group during CBP.

Discussion

The hemostatic effect of CBP on platelet function and composition is still unclear.

Many authors have postulated changes in platelet function as reflected by alterations in surface expression of GP during CBP and platelet agglutination and aggregation capacity.[2,5,6,9-16,18,19,21-24,26-28] Other authors have reported no changes in the surface expression of platelet GP complexes, and a slight decrease in platelet reactivity *in vitro*, attributed mainly to technical problems.[1,7] In relation to aprotinin, most authors showed a beneficial effect of high-doses[6,9-22] and low-doses of aprotinin, reflected by a protective effect on GP complexes that correlated with a beneficial effect on agglutination[25] or aggregation capacity of platelets.[9,28] In contrast, some authors have demonstrated that high-doses of aprotinin have no effect on platelet function and *in vitro* reactivity.[7,27] Studies comparing two aprotinin dosages[9,15,24] have reported changes in platelet function during CBP, although results are contradictory. Boldt *et al.* concluded that neither low doses nor high doses of aprotinin given to children undergoing CBP in cardiac operations produced improvements,[24]

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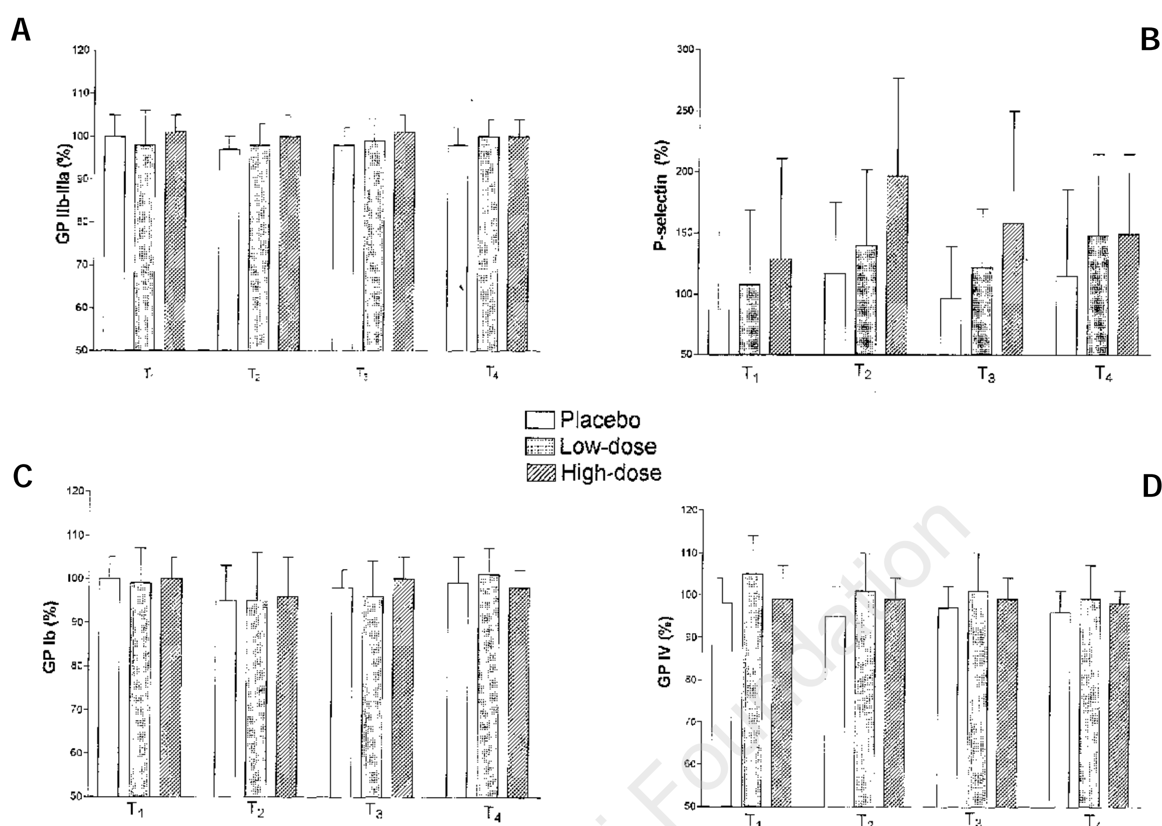

Figure 1. Comparison of expression of platelet surface glycoproteins, determined by a flow cytometric method, between groups during and after CBP (T1, immediately before operation; T2, at the end of CBP but before protamine administration; T3, 1 hour after protamine neutralization; T4, 18-24 hours after operation). A: Surface expression of GPIIb-IIIa receptor. B: expression of P-selectin receptor. C: Expression of GPIb receptor. D: Expression of GP-IV receptor. Results are shown as mean ± standard error.

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but Mohr *et al.* and Van Ouveren *et al.* reported that platelet function was improved by aprotinin.[9,15]

Another question to take into consideration is the potential methodologic problems related to sample processing in different assays.[29] Despite the fact that we used a flow cytometric method of assessing formaldehyde fixed platelets, our results reached similar conclusions concerning changes of platelet surface receptors during CBP as those of Kestin *et al.*, using whole blood samples, and other authors.[1,26]

Regarding the theory that platelets are affected in the first 5 minutes of CBP, and that a pump-prime-only dose of aprotinin could diminish the initial impairment of platelet hemostatic function, we did not find, in our study, any evidence of an initial benefit of a pump-prime-only aprotinin dose on platelet function.[1,9]

In this study, we demonstrated that during CBP, platelet surface expression of GPIIb-IIIa, GPIV and GPIb-X did not change in any group, but that P-selectin increases in all groups. By contrast, platelet reactivity *in vitro* showed significantly deficient platelet aggregation in response to agonist in patients treated with aprotinin or not treated. Nor did we found any correlation between decrease in agglutination capacity of platelets and levels of GPIb-X and aggregation capacity of platelets and levels of GPIIb-IIa complexes. Thus, in this study different doses of aprotinin were not observed to protect adhesive and aggregation capacity of platelets.[1,5] Moreover, significant blood loss reduction and decrease of D-dimer levels in patients treated with aprotinin were not correlated with an improvement in platelet function.

In view of these results, we can conclude that changes in platelet function are related to a significant decrease in agglutination and aggregation capacity throughout CBP, reflecting the hemostatic effect of CBP on platelet function, and not an effect on surface expression of GP complexes implicated in adhesion and aggregation platelet function. The hemostatic effect of aprotinin, regardless of the use of high or low doses, is not mediated by protection of platelet function.

New approaches should be studied in order to assess the alteration of platelet function during CBP and the possibility of introducing other drugs with protective action on platelet function, in addition to the use of aprotinin, during CBP.

Contributions and Acknowledgments

AS designed the study, was responsible for the data management and prepared the manuscript.

JM also designed the study and supervised the study. EM performed the cytometric analyses, collected data and revised the manuscript. AO was responsible for data analysis. JM collected all biological data. HL collaborated in patient care and collecting data. JS supervised the manuscript. JF revised the manuscript and gave final approval for its submission. The order in which the names appear is based on the fraction of the total work performed. We wish to thank to George von Knorring for his assistance in the preparation of the manuscript.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received September 14, 1999; accepted December 13, 1999.

Potential Implications for clinical practice

- New approaches should be studied in order to assess the alteration on platelet function during the CBP and the possibility of introducing other drugs with protective action on platelet function, in addition to the use of aprotinin, during CBP.

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