Combined administration of nitric oxide gas and iloprost during cardiopulmonary bypass reduces platelet dysfunction: A pilot clinical study

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Background: Thrombocytopenia and platelet dysfunction are major mechanisms of cardiopulmonary bypass–induced postoperative hemorrhage. This study evaluated the effects of low amounts of nitric oxide, iloprost (prostacyclin analog), and their combination administered directly into the oxygenator on platelet function, platelet-leukocyte interactions, and postoperative blood loss in patients undergoing coronary artery bypass grafting.

Methods: Blood samples from 41 patients randomized to the control, nitric oxide (20 ppm), iloprost (2 ng · kg⁻¹ · min⁻¹), or nitric oxide plus iloprost groups were collected during cardiopulmonary bypass. Platelets and leukocytes were enumerated. Platelet membrane glycoprotein Ib and glycoprotein IIb/IIIa, P-selectin, platelet-derived microparticles, leukocyte CD11b/CD18 (Mac-1), and platelet-leukocyte aggregate were quantified by means of flow cytometry. Collagen and thrombin receptor-activating peptide–induced platelet aggregation in whole blood was analyzed by means of aggregometry.

Results: Both nitric oxide or iloprost attenuated cardiopulmonary bypass–induced thrombocytopenia, reduction of glycoprotein Ib and glycoprotein IIb levels, translocation of P-selectin, microparticle formation, Mac-1 upregulation, and suppression of collagen-induced aggregation. Nitric oxide plus iloprost was significantly more effective in preventing thrombocytopenia, microparticle formation, and P-selectin translocation. Moreover, this treatment preserved thrombin receptor-activating peptide–induced aggregation, which was not rescued by single treatments. Both nitric oxide and nitric oxide plus iloprost attenuated postoperative blood loss.

Conclusions: Nitric oxide plus iloprost reduced the deleterious effects of cardiopulmonary bypass, such as thrombocytopenia, platelet activation, platelet-leukocyte aggregate formation, and suppression of platelet aggregative responses. The reduced postoperative bleeding observed with this treatment suggests that this is a new and clinically feasible therapeutic option for patients subjected to cardiopulmonary bypass.

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The development of cardiopulmonary bypass (CPB) has been one of the most important advancements in cardiac surgery. However, CPB is associated with thrombocytopenia, which is caused by activation and consumption of platelets, and leukocytosis. These reactions are responsible for some adverse effects of the procedure, including bleeding and systemic inflammation.^{1,2} The pathophysiologic effects of platelet activation induced by CPB are still poorly understood. However, formation of platelet-leukocyte aggregates (PLAs) in the presence of artificial surfaces of

TABLE 1. Patient demographics and postoperative clinical parameters

Characteristics	Control	NO	PGI ₂	NO + PGI ₂	P value
Sample size	18	8	10	5	—
Age (y)	61 ± 12	64 ± 8	65 ± 11	63 ± 9	NS
Weight (kg)	86.2 ± 3.7	91.2 ± 8.3	89.8 ± 5.8	85.6 ± 12.3	NS
Height (cm)	174 ± 8.2	175 ± 12.8	171 ± 10.4	170 ± 9	NS
NYHA class	2.2 ± 0.8	2.6 ± 0.5	2.4 ± 0.7	2.6 ± 0.5	NS
Number of CAD risk factors per patient	2.2	2.6	2.6	2.8	NS
CAD risk factors					
Diabetes, n (%)	8 (44)	4 (50)	6 (60)	3 (60)	NS
Hypertension, n (%)	10 (56)	5 (62)	6 (60)	3 (60)	NS
Hyperlipidemia, n (%)	5 (28)	3 (37)	3 (30)	2 (40)	NS
History of smoking, n (%)	12 (67)	6 (75)	7 (70)	4 (80)	NS
Hyperuremia, n (%)	4 (22)	2 (25)	2 (20)	1 (20)	NS
Alcohol, n (%)	2 (11)	1 (13)	2 (20)	1 (20)	NS
CCS class	2.6 ± 0.9	3 ± 0.7	2.7 ± 0.5	2.7 ± 0.6	NS
Ejection fraction (%)	51 ± 9	43 ± 7	46 ± 15	45 ± 11	NS
Pump time (min)	104 ± 16	99 ± 29	80 ± 13	83 ± 20	NS
Clamp time (min)	68 ± 18	52 ± 14	49 ± 22	48 ± 12	NS
Flow (L/min)	4.8 ± 0.3	4.6 ± 0.4	4.7 ± 0.4	4.2 ± 0.5	NS
p max					
1 min	121 ± 110	123 ± 15	144 ± 47	105 ± 27	NS
30 min	118 ± 87	128 ± 21	138 ± 20	100 ± 20	NS
60 min	97 ± 33	129 ± 22	144 ± 26	104 ± 27	NS
End of pump	92 ± 27	128 ± 23	134 ± 20	106 ± 23	NS
No. of grafts	3.3 ± 0.6	3.3 ± 0.7	3.4 ± 0.5	3.3 ± 0.5	NS
Inotropic support					
Epinephrine (μg · kg ⁻¹ · min ⁻¹)	0.6 ± 0.5	0.3 ± 0.6	0.4 ± 0.6	0.4 ± 0.3	NS
Norepinephrine (μg · kg ⁻¹ · min ⁻¹)	0.3 ± 0.2	0.2 ± 0.4	0.3 ± 0.2	0.4 ± 0.4	NS
Hemoglobin after CPB (g/dL)	10.3 ± 4.04	10.6 ± 4.3	9.8 ± 3.1	9.6 ± 3.7	NS
Intraoperative RBCs (units/patient)	0.9 ± 0.6	1.2 ± 0.8	1.1 ± 0.7	1.3 ± 1	NS
Postoperative RBCs given (units/patient)	3.4 ± 1.6	2.8 ± 1.1	2.7 ± 1.9	2.8 ± 1.2	NS
Postoperative FFP given (units/patient)	1.8 ± 0.7	1.3 ± 0.4	1.7 ± 1.1	1.6 ± 1	NS
Postoperative platelets given (units/patient)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	NS
Time to extubation (h)	12 ± 3.3	10.4 ± 6.8	9 ± 3	10 ± 1.3	NS
ICU stay (h)	22.8 ± 4.2	22.5 ± 1.4	27 ± 4	21.7 ± 1.3	NS
Methemoglobin (%)	0.26 ± 0.1	0.31 ± 0.1	0.28 ± 0.14	0.29 ± 0.12	NS

NO, Nitric oxide; PGI₂, prostacyclin; NYHA, New York Heart Association; CAD, coronary artery disease; CCS, Canadian Cardiovascular Society; CPB, cardiopulmonary bypass; RBCs, red blood cells; FFP, fresh frozen plasma; ICU, intensive care unit; NS, not significant.

the CPB circuit might be an important mechanism of thrombotic and inflammatory complications caused by CPB. The development of selective therapeutic strategies to preserve the function of platelets and leukocytes could reduce postoperative hemorrhage, systemic inflammation, and vascular dysfunction, with subsequent requirement for transfusion of blood products and inotropes. This might have a significant effect on patient morbidity and mortality.³ Nitric oxide (NO) gas, prostacyclin (PGI₂), and iloprost, a stable analog of PGI₂, are potent inhibitors of platelet activation in vitro and in vivo⁴; however, their effects on specific platelet and leukocyte receptors have not been comprehensively studied in the setting of CPB. We designed a pilot clinical study to investigate changes in platelet and leukocyte functions in patients subjected to CPB. Furthermore, we studied the

potential protective effects of NO and iloprost on platelets and leukocytes when administered directly into the oxygenator of the CPB circuit. Moreover, we examined clinical outcome parameters, such as thrombocytopenia and postoperative bleeding, to account for the pharmacodynamic effects of both drugs. The results of this study show that combined therapy with NO gas (20 ppm) and iloprost (2 ng · kg⁻¹ · min⁻¹) decreased circuit-induced platelet dysfunction and reduced postoperative bleeding in patients subjected to CPB.

Methods

Reagents

Collagen and thrombin receptor-activating peptide (TRAP) were purchased from Chronolog (Havertown, Pa) and Sigma (St Louis,

Figure 1. Effects of NO and iloprost on CPB-induced changes in platelet and leukocyte numbers. * $P < .05$, treatments versus control; # $P < .05$, NO plus iloprost versus single treatment (analysis of variance with Dunnett test).

Mo), respectively. Fluorescein isothiocyanate (FITC)-conjugated monoclonal mouse antibodies (MoAbs) directed against glycoprotein (GP) IIb (CD41-FITC) and phycoerythrin (PE)-conjugated MoAbs directed against GPIb (CD42-PE) were from DAKO Diagnostics Canada Inc, Mississauga, Ontario, Canada. MoAbs against activated GPIIb (PAC-1-FITC), P-selectin (CD62P-PE), leukocyte common antigen (CD45-FITC), and Mac-1 (CD11b-PE) were from Becton Dickinson (Franklin Lakes, NJ).

Patient Selection and Experimental Design

The Health Research Ethics Board of the University of Alberta, Edmonton, Canada, approved this study. Informed consent was obtained from all patients before the study. Forty-one patients were randomly assigned to one of the 4 groups: (1) routine CPB (control); (2) routine CPB plus NO (20 ppm) administered into the membrane oxygenator; (3) routine CPB plus iloprost ($2 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) administered into the blood phase of the oxygenator; and (4) routine CPB plus NO plus iloprost.

All patients included were elective patients scheduled for coronary artery bypass grafting. Excluded were patients undergoing combined procedures and reoperation. Further exclusion criteria were evidence for impaired platelet function and coagulation disorders, chronic inflammatory diseases, and renal failure. It was ensured that platelet inhibitor drugs, such as aspirin or clopidogrel, were discontinued at least 7 days before the operation.

NO (PulmNOx Medical Inc, Tofield, Alberta, Canada) was administered into the membrane oxygenator through a Y connector during the period of extracorporeal circulation. Iloprost (Cayman Chemical Company, Ann Arbor, Mich) was infused into the oxygenator starting from the initiation of CPB and was maintained throughout the duration of CPB. The doses of NO and iloprost were selected on the basis of preliminary studies showing significant effects of these drugs on platelet function without alterations of hemodynamics (mean arterial blood pressure) or increase in vasopressor requirement.

For safety reasons, the staff in the operating room, including surgeons, perfusionists, and anesthesiologists, was not blinded to

the study. In addition, the exhaust, including the outflow from the extracorporeal circuit, was removed from the operating room through central air conditioning. However, the basic scientists involved in the sample collection, processing, and data analysis were blinded to the treatment and control groups.

Anesthesia was identical in each group, according to a standard protocol of the institution, except that aprotinin and all nitrovasodilators were excluded.

The surgical procedure was performed through a midline sternotomy, with systemic heparinization and an activating clotting time well above 400 seconds. All extracorporeal circuits used were noncoated, were identical in each patient, and consisted of a membrane oxygenator, arterial filter, and colloidal priming (1400 mL). No patient received aprotinin or other proteinase inhibitors during the study period. Cardioplegic arrest was performed with blood cardioplegia.⁵ The temperature during bypass grafting was between 32°C and 34°C , and rewarming was initiated after completion of the last peripheral anastomosis. Reperfusion was performed with whole blood by means of aortic declamping. The demographic characteristics, clinical parameters of the patients, number of grafts, reperfusion times, and aortic crossclamp times are given in Table 1.

Whole blood was withdrawn from the arterial line at 2 time points: at the onset of CPB (prepump; ie, 5 minutes after heparin administration) and at the termination of CPB (postpump; ie, before protamine administration). All blood samples were collected with 3.15% wt/vol trisodium citrate preparation (9:1 vol/vol) and counted with MicroDiff 16 (Coulter Electronics, Hialeah, Fla).

The 2 time points selected for analysis of platelet aggregation and flow cytometry were chosen to study the maximal effects of CPB on platelet function. We hypothesized that this protocol would enable us to detect the maximal difference between the control and treatment groups. In addition, technically it was impossible to perform any additional time point analysis on fresh blood samples because processing the samples would have ex-

Figure 2. Effects of NO and iloprost on CPB-induced changes in platelet receptors (GPIIb and total and active GPIIb) and MPs. * $P < .05$, treatments versus control; # $P < .05$, NO plus iloprost versus single treatment (analysis of variance with Dunnett test).

ceeded the duration of bypass. All data affected by hemodilution were corrected for it.

Platelet Aggregation

Platelet aggregation was measured by means of whole blood aggregometry with a whole blood ionized calcium lumi-aggregometer (Chronolog). Changes in impedance (ohm) were recorded as an index of aggregation. Because blood aggregation depends on many factors, including red blood cell counts, white blood cell counts, and fibrinogen and plasminogen concentrations in the plasma, the results were expressed as a percentage of the maximal aggregative response of each patient.

Flow Cytometry

To determine the relative abundance of GPIIb, total-active GPIIb, and P-selectin, samples were incubated for 20 minutes with saturating concentrations of FITC- or PE-conjugated antibodies, such as CD42-PE, CD41-FITC, PAC-1-FITC, or CD62P-PE. The platelet population was gated by using forward- and side-scatter parameters. Fluorescence intensity was analyzed with logarithmic scale and CELLQUEST software. The quantification of GPIIb, total-active GPIIb, and P-selectin levels was expressed as the mean fluorescence intensity (MFI) from 10,000 individual events. For microparticle (MP) analysis, samples were incubated with CD42-PE MoAbs, and the MP population was distinguished by the forward-scatter cutoff that was set to the immediate left of the platelet population. MPs were

reported as the percentage of PE-positive cells. Mac-1 abundance was analyzed by means of dual-labeling leukocytes with CD45-FITC and CD11b-PE MoAbs, and the MFI was determined from the FITC-positive cells. The PLA formation was expressed as $10^9/L$ and was assayed by double-labeling samples with CD45-FITC and CD62P-PE MoAbs, quantifying the percentage of leukocytes exhibiting CD62P fluorescence, and multiplying the percentage of PLA by the number of leukocytes in the samples.

Statistical Analysis

All results were given as means $\pm$ SEM derived from n determinations and were analyzed by using analysis of variance with the Bonferroni or Dunnett tests, where appropriate, with Prism software (GraphPad Software, Inc, San Diego, Calif).

Results

Effects of CPB on Platelet and Leukocyte Functions

Before the operation, leukocyte and platelet numbers were $6.5 \pm 0.5 \times 10^9$ and $2.05 \pm 0.25 \times 10^{11}/L$, respectively. The surgical procedure with CPB resulted in a significant reduction of platelet numbers and an increase in leukocyte numbers when compared with prepump values (Figure 1). These changes were associated with increased platelet and leukocyte activation. In blood samples collected before the operation, the levels of GPIIb, total GPIIb, activated GPIIb,

Figure 3. Effects of NO and iloprost on CPB-induced changes in Mac-1, P-selectin, and PLA. * $P < .05$ versus control; # $P < .05$ NO plus iloprost versus single treatment (analysis of variance with Dunnett test).

P-selectin, and Mac-1 were 850 ± 40 , 650 ± 40 , 4.5 ± 0.2 , 14.8 ± 0.3 , and 310 ± 20 MFI, respectively. In addition, 400 ± 20 MPs were detected in 10,000 events, and there was 2.5 ± 0.1 PLA per 10^8 platelets. Platelet activation was evidenced by downregulation of GPIb levels and reduction of total and active GPIIb levels. In addition, the levels of MPs were increased (Figure 2). Leukocytes became activated, as shown by a significant increase of Mac-1 levels (Figure 3). Furthermore, there was a significant upregulation of platelet-leukocyte interactions as P-selectin and PLA levels were increased (Figure 3).

The markers of platelet and leukocyte activation in vivo were accompanied by decreased platelet aggregation induced by collagen and TRAP ex vivo. This was shown by the right shift of the respective concentration-response curves (Figure 4, A) and the corresponding increase in median effective concentration (EC_{50}) values from 1.1 ± 0.1 to 1.9 ± 0.1 mg/L (collagen) and from 5.9 ± 0.2 to 10.0 ± 0.1 μ mol/L (TRAP; $P < .05$).

Effects of NO on CPB-induced Platelet Activation and Platelet-leukocyte Activation

In the presence of NO, platelet counts remained reduced; however, this reduction was significantly attenuated com-

pared with levels in the nontreated group (Figure 1). The NO treatment failed to prevent the significant increase in leukocyte numbers (Figure 1).

The panel of CPB-induced markers of platelet activation, such as GPIb and GPIIb downregulation and MP formation, were significantly attenuated by NO (Figure 2). In addition, there was decreased leukocyte activation and formation of PLA (Figure 3), as demonstrated by reduced Mac-1, P-selectin, and PLA levels.

Platelet aggregation showed that although the aggregative responses to collagen were preserved by the NO treatment (EC_{50} postpump value of 1.2 ± 0.1 vs prepump value of 1.3 ± 0.2 mg/L, $P > .05$), TRAP-induced aggregation remained decreased (EC_{50} postpump value of 14.2 ± 2.8 vs prepump value of 5.6 ± 0.2 μ mol/L, $P < .05$).

Effects of Iloprost on CPB-induced Platelet-leukocyte Activation

With the use of iloprost, platelet numbers were reduced by $14\% \pm 2\%$, which was less than those in control subjects. Iloprost exerted no significant effects on leukocytosis (increase by $92\% \pm 25\%$, Figure 1).

However, iloprost attenuated the effects of CPB on GPIb, GPIIb, activated GPIIb, MP, and P-selectin. In con-

Figure 4. CPB-induced changes in platelet aggregation to collagen and TRAP (A) and the effects of NO plus iloprost (B). * $P < .05$, treatments versus control (analysis of variance with Dunnett test).

trast, the treatment enhanced the effects of CPB on Mac-1 and PLA (Figures 2 and 3). Aggregation to collagen was preserved (EC_{50} postpump value of 1.2 ± 0.2 vs prepump value of 1.4 ± 0.1 mg/L, $P > .05$), whereas that to TRAP remained decreased (EC_{50} postpump value of 13.8 ± 1.1 vs prepump value of 6.3 ± 0.3 μ mol/L, $P > .05$).

Effects of NO plus Iloprost on CPB-induced Platelet-leukocyte Activation

The combination therapy effectively prevented thrombocytopenia but did not prevent leukocytosis (Figure 1). The drugs attenuated the effects of CPB on GPIb, total GPIIb, MP, P-selectin, Mac-1, and PLA (Figures 2 and 3).

Interestingly, aggregation to collagen and TRAP were well preserved (Figure 4, B).

Effects of NO and Iloprost on Chest Tube Loss

Figure 5 shows the effects of treatments with NO and iloprost on chest tube blood loss after cardiac surgery. Both NO and NO plus iloprost, but not iloprost alone, significantly attenuated this blood loss.

Discussion

We investigated platelet- and leukocyte-related mechanisms of CPB-induced postoperative bleeding. A very high effectiveness of combinations of NO and PGI_2 in attenuating platelet activation in vitro^{4,6} prompted us to design a pilot clinical study to evaluate the effects of these inhibitors on platelet-leukocyte function and postoperative bleeding in patients subjected to coronary revascularization with CPB.

The major novel findings of our investigation showed that the combined treatment with NO and iloprost (1) abol-

Figure 5. Chest tube blood loss after CPB and the effects of NO and iloprost. * $P < .05$, treatments versus control (analysis of variance with Dunnett test).

ished the CPB-induced thrombocytopenia, (2) prevented the loss of platelet aggregation to collagen and TRAP, and (3) reduced postoperative bleeding.

CPB and Platelet-leukocyte Dysfunction

The increased tendency for hemorrhage in patients undergoing cardiac surgery with CPB is likely to be multifactorial; it involves thrombocytopenia, increased coagulation, fibrinolysis, complement activation, and inflammation. In addition, it is known that pump and clamp times, as well as flow rates, are important determinants of platelet and leukocyte activation and function. In this regard a recent study by Greilich and colleagues⁷ showed that prolongation of CPB is related to increasing degrees of platelet dysfunction and that reductions in platelet contractile force are related to decreases in platelet adhesion receptors and early postoperative blood loss. This pathologic scenario is often associated with the postoperative need for transfusion of blood products and the requirement for inotropic support, both of which are risk factors for morbidity and mortality.²

Previous studies and the current investigation show that thrombocytopenia is a major contributing factor to CPB-induced bleeding.^{2,8,9} Early hemodilution caused by the crystalloid fluid for priming of the extracorporeal circuit might partially explain this phenomenon. However, Holloway and coworkers⁹ suggested that the mechanical disruption of platelets by shear forces (oxygenator) and increased platelet adhesion to the extracorporeal membrane and sequestration in organs both contribute to the true decrease in circulating platelet numbers. Our study provides a compelling indication for in-circuit activation of platelets. This is evidenced by a large increase in MP formation that derives from the membrane of activated platelets,¹⁰ downregulation of GPIb that results from its internalization,¹¹ and the translocation of platelet α -granule P-selectin to the surface of activated platelets.¹² Downregulation of total and active

GPIIb provides a strong rationale for the proposal that circuit-activated platelets are exhausted and no longer capable of responding with aggregation to hemostatic regulators in vivo.¹ This hemostatic defect can be exposed by ex vivo adhesion and aggregation studies that demonstrated impaired platelet reactivity to collagen, thrombin,¹³⁻¹⁵ and, as shown in the current study, TRAP. Therefore the major mechanisms of CPB-related postoperative bleeding are both functional (platelet reactivity) and quantitative (platelet count) defects.^{13,16}

We and others have shown that thrombocytopenia is accompanied by leukocytosis that is associated with the inflammatory response.¹⁷ The surfaces of CPB systems (ie, oxygenator and tubing) are recognized as foreign by the immune system, which subsequently activates a cytokine cascade.¹⁸ Cytokines initiate systemic inflammatory reactions, leading to leukocyte recruitment and activation and generation of oxidants and radicals, such as superoxide. Clinically, these reactions result in systemic vasodilation, increased vascular permeability, and organ dysfunction.

The role of platelets in CPB-induced inflammation is not clear; however, PLA might play a critical role in this process. Indeed, activated platelets translocate P-selectin to their surface, whereas Mac-1 (CD11b/CD18) is upregulated by activated leukocytes. The interactions of these adhesion receptors with their ligands¹⁹⁻²¹ facilitate PLA formation, endothelial adhesion, and inflammatory leukocyte extravasation. Increased Mac-1 and P-selectin levels in conjunction with enhanced PLA during CPB are well evidenced by our study. Therefore, simultaneous activation of both platelets and leukocytes with platelet adhesion, aggregation, and PLA can all contribute to circuit-induced thrombocytopenia, inflammatory responses, and increased postoperative blood loss. It is important to note that in the present study no differences between the control and treatment groups with regard to pump and clamp times, as well as flow rates, were observed (Table 1).

Effects of NO or Iloprost on CPB

We also demonstrated the protective effects of NO and iloprost on platelet function. Both drugs protected platelets from activation, as evidenced by attenuation of thrombocytopenia and inhibition of changes in platelet receptors. Because NO and PGI₂ and its analog iloprost are well-known regulators of platelet activation and inhibit platelet adhesion and aggregation at various levels of the activating cascade,⁴ the effectiveness of these drugs in limiting platelet damage is not surprising.

Indeed, the beneficial effects of NO and PGI₂ have also been demonstrated in other studies. Aren and associates²² showed that PGI₂ infusion into the bypass circuit reduced plasma β -thromboglobulin levels, reflecting inhibition of platelet activation, although the dosage used ($50 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)

was much higher than in our study ($2 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$). Mellgren and colleagues²³ demonstrated higher platelet counts in experimental perfusion circuits treated with NO (15–75 ppm); however, platelet function was not measured in this study. Inhaled NO downregulated P-selectin, platelet aggregation, and fibrinogen binding in patients with severe adult respiratory distress syndrome,²⁴ but the concentration of NO (100–884 ppm) was again much higher than in our study (20 ppm). Furthermore, extracorporeal circuits coated with NO release polymers reduced platelet consumption in experimental animals.²⁵ The protective effects of NO and PGI₂ in CPB, however, are not without considerable debate. PGI₂ infusion might cause hypotension,²⁶ suggesting that the use of high amounts of these potent platelet inhibitors is likely to affect the hemodynamic balance by increasing vasodilatation and vascular permeability. Therefore the use of small (nonvasodilator) amounts of NO and iloprost (that proved to be efficacious in our study) applied directly to the oxygenator might be preferable in the quest to limit the CPB-induced platelet activation and damage.

Interestingly, the iloprost treatment increased Mac-1 levels in white blood cells. In contrast to platelets, where stimulation with antiaggregative prostaglandins, such as prostacyclin and prostaglandin D₂, invariably leads to downregulation of integrin function, the effects of prostaglandins on white blood cells appear to be more complex. Indeed, prostaglandin D₂ stimulates chemotaxis and Mac-1 expression²⁷ in eosinophils through activation of a novel DP2 receptor. Thus, the stimulator effects of iloprost on Mac-1 could reflect activation of the DP2 receptor in white blood cells.

Effects of NO plus Iloprost on CPB

The major interest of our study has been focused on the combined use of NO and iloprost during CPB. The *in vitro* studies clearly showed that NO and PGI₂ synergize with each other as inhibitors of platelet aggregation and inducers of deaggregation. The current study has shown that the most striking advantage of using this combination of low doses of NO and iloprost is total prevention of thrombocytopenia and normalization of circuit-induced aggregative dysfunction. NO plus iloprost also reduced postoperative blood loss; however, in this action NO was as efficacious as the combination. The protective effects afforded by NO plus iloprost correlated well with reduction of MP and P-selectin levels. However, no additional protection over that seen with single-drug treatment could be detected with GPIb and GPIIb measurements. The reason or reasons for this discrepancy are not known.

Mechanisms of Platelet-protective Effects of NO and Iloprost

NO and iloprost inhibit platelet activation by stimulating the intraplatelet levels of cyclic guanosine monophosphate

(cGMP) and cyclic adenosine monophosphate (cAMP), respectively.⁴ Indeed, increase of intracellular cAMP and cGMP levels is the most potent endogenous mechanism of platelet inhibition. Cyclic nucleotides control a number of platelet functions, such as inhibition of intracellular Ca²⁺ increase, cytoskeletal reorganization, integrin receptor activation, granule secretion, surface molecule expression, and mitogen-activated protein kinase function.²⁸ All these effects result in inhibition of platelet activation. There is also evidence that the cAMP and cGMP pathways cross-talk to inhibit platelet activation. Cyclic GMP-mediated inhibition of cAMP hydrolysis through reduction of phosphodiesterase 3 activity might be one of the mechanisms that account for the synergistic interactions between NO and iloprost on platelets.^{28,29}

As with single-compound treatment, the combined therapy exerted no significant effects on leukocytosis and only weakly affected Mac-1 and PLA levels. Thus other pharmacologic strategies should be used to decrease further circuit-induced inflammatory reactions.

Clinical Implications of the Present Study

The most important clinical aspect to be derived from the present findings is the potential for significant reduction in postoperative bleeding by means of preservation of platelet function. This might be even more pronounced in the setting of prolonged pump time or in patients receiving long-term extracorporeal support, including extracorporeal membrane oxygenation and assist devices. Indeed, Jacobson³⁰ has recently pointed out that NO is a potent platelet protectant against a significant reduction in platelet number and function, as observed in patients requiring long-term mechanical support.

Another important aspect of the use of the combination of NO and iloprost is cost-effectiveness. In our center the average cost for such therapy per one patient is \$25 for NO and \$15 for iloprost, assuming a pump time of 60 minutes. Given the potential saving effect on blood products with this treatment strategy, the overall cost might be reduced.

Study Limitations

We acknowledge that there are limitations of this pilot clinical study. The first is the relatively small number of patients in the treatment groups. However, our study was of sufficient statistical power to delineate differences in platelet function and platelet-leukocyte interactions between the control and treatment groups. Furthermore, the conclusions of our study are based not on the analysis of single variable but on the use of a panel of molecular (GP receptors, MPs, P-selectin, and Mac-1 levels), functional (platelet aggregation and platelet-leukocyte aggregation), and clinical (platelet and leukocyte numbers and postoperative blood loss)

variables. This analysis clearly shows the protective effects on platelets and the resultant clinical benefits (reduction of thrombocytopenia and postoperative blood loss) of nonvasodilator amounts of NO and iloprost.

Second, in this study we focused only on the acute effects of NO and iloprost on blood cells. It is possible that longer treatment regimens would have additional beneficial effects on postoperative bleeding or other thrombotic and inflammatory complications of CPB. In addition, in the present study no differences were observed between groups with regard to the administration of blood products. One reason might be that the patients included in our study were elective patients, excluding those scheduled for repeat operations and those expected to have long pump times. Thus the present study was performed in a selected group of patients not at high risk for major bleeding disorders. However, one can speculate that the finding of reduced chest tube loss and the platelet-sparing effect with NO and iloprost, as shown in the present study, might be even more pronounced in larger trials with higher-risk patients and prolonged CPB procedures, resulting in reduction of transfusion requirements.

In summary, the present study demonstrates, for the first time, that combined treatment with low amounts of NO (20 ppm) or iloprost ($2 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), when delivered into the oxygenator, reduced thrombocytopenia, normalized platelet aggregation, and attenuated postoperative blood loss, providing a safe method to decrease bleeding in patients subjected to CPB.

References

- Weerasinghe A, Taylor KM. The platelet in cardiopulmonary bypass. *Ann Thorac Surg*. 1998;66:2145-52.
- Woodman RC, Harker LA. Bleeding complications associated with cardiopulmonary bypass. *Blood*. 1990;76:1680-97.
- Asimakopoulos G. Systemic inflammation and cardiac surgery: an update. *Perfusion*. 2001;16:353-60.
- Jurasz P, Radomski A, Sawicki G, Mayers I, Radomski MW. Nitric oxide and platelet function. In: LJ I, editor. Nitric oxide biology and pathobiology. San Diego: Academic Press; 2000. p. 823-40.
- Buckberg GD. Antegrade/retrograde blood cardioplegia to ensure cardioplegic distribution: operative techniques and objectives. *J Card Surg*. 1989;4:216-38.
- Radomski MW, Palmer RM, Moncada S. The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br J Pharmacol*. 1987;92:639-46.
- Greilich PE, Brouse CF, Beckham J, Jessen ME, Martin EJ, Carr ME. Reductions in platelet contractile force correlate with duration of cardiopulmonary bypass and blood loss in patients undergoing cardiac surgery. *Thromb Res*. 2002;105:523-9.
- Despotis GJ, Joist JH, Goodnough LT. Monitoring of hemostasis in cardiac surgical patients: impact of point-of-care testing on blood loss and transfusion outcomes. *Clin Chem*. 1997;43:1684-96.
- Holloway DS, Summaria L, Sandesara J, Vagher JP, Alexander JC, Caprini JA. Decreased platelet number and function and increased fibrinolysis contribute to postoperative bleeding in cardiopulmonary bypass patients. *Thromb Haemost*. 1988;59:62-7.
- Michelson AD. Flow cytometry: a clinical test of platelet function. *Blood*. 1996;87:4925-36.
- Radomski A, Stewart MW, Jurasz P, Radomski MW. Pharmacological characteristics of solid-phase von Willebrand factor in human platelets. *Br J Pharmacol*. 2001;134:1013-20.
- Brown AS, Moro MA, Masse JM, Cramer EM, Radomski M, Darley-Usmar V. Nitric oxide-dependent and independent effects on human platelets treated with peroxynitrite. *Cardiovasc Res*. 1998;40:380-8.
- Kabakibi A, Vamvakas EC, Cannistraro PA, Szczepiorkowski ZM, Laposata M. Collagen-induced whole blood platelet aggregation in patients undergoing surgical procedures associated with minimal to moderate blood loss. *Am J Clin Pathol*. 1998;109:392-8.
- Uthoff K, Zehr KJ, Geerling R, Herskowitz A, Cameron DE, Reitz BA. Inhibition of platelet adhesion during cardiopulmonary bypass reduces postoperative bleeding. *Circulation*. 1994;90(suppl):II269-74.
- Ferraris VA, Ferraris SP, Singh A, Fuhr W, Koppel D, McKenna D, et al. The platelet thrombin receptor and postoperative bleeding. *Ann Thorac Surg*. 1998;65:352-8.
- Ray MJ, Hawson GA, Just SJ, McLachlan G, O'Brien M. Relationship of platelet aggregation to bleeding after cardiopulmonary bypass. *Ann Thorac Surg*. 1994;57:981-6.
- Despotis GJ, Alsoufiev A, Hogue CW, Zoys TN, Goodnough LT, Santoro SA, et al. Evaluation of complete blood count results from a new, on-site hemocytometer compared with a laboratory-based hemocytometer. *Crit Care Med*. 1996;24:1163-7.
- Tonnesen E, Christensen VB, Toft P. The role of cytokines in cardiac surgery. *Int J Cardiol*. 1996;53(suppl):S1-10.
- Smith CW, Marlin SD, Rothlein R, Toman C, Anderson DC. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. *J Clin Invest*. 1989;83:2008-17.
- Simon DI, Chen Z, Xu H, Li CQ, Dong J, McIntire LV, et al. Platelet glycoprotein Ib/alpha is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J Exp Med*. 2000;192:193-204.
- Moore KL. Structure and function of P-selectin glycoprotein ligand-1. *Leuk Lymphoma*. 1998;29:1-15.
- Aren C, Feddersen K, Radegran K. Effects of prostacyclin infusion on platelet activation and postoperative blood loss in coronary bypass. *Ann Thorac Surg*. 1983;36:49-54.
- Mellgren K, Friberg LG, Mellgren G, Hedner T, Wennmalm A, Wadenvik H. Nitric oxide in the oxygenator sweep gas reduces platelet activation during experimental perfusion. *Ann Thorac Surg*. 1996;61:1194-8.
- Gries A, Bode C, Peter K, Herr A, Bohrer H, Motsch J, Martin E. Inhaled nitric oxide inhibits human platelet aggregation, P-selectin expression, and fibrinogen binding in vitro and in vivo. *Circulation*. 1998;97:1481-7.
- Annich GM, Meinhardt JP, Mowery KA, Ashton BA, Merz SI, Hirschl RB, et al. Reduced platelet activation and thrombosis in extracorporeal circuits coated with nitric oxide release polymers. *Crit Care Med*. 2000;28:915-20.
- DiSesa VJ, Huval W, Lelcuk S, Jonas R, Maddi R, Lee-Son S, et al. Disadvantages of prostacyclin infusion during cardiopulmonary bypass: a double-blind study of 50 patients having coronary revascularization. *Ann Thorac Surg*. 1984;38:514-9.
- Monneret G, Gravel S, Diamond M, Rokach J, Powell WS. Prostaglandin D2 is a potent chemoattractant for human eosinophils that acts via a novel DP receptor. *Blood*. 2001;98:1942-8.
- Schwarz UR, Walter U, Eigenthaler M. Taming platelets with cyclic nucleotides. *Biochem Pharmacol*. 2001;62:1153-61.
- Feil R, Lohmann SM, de Jonge H, Walter U, Hofmann F. Cyclic GMP-dependent protein kinases and the cardiovascular system: insights from genetically modified mice. *Circ Res*. 2003;93:907-16.
- Jacobson J. Nitric oxide: platelet protectant properties during cardiopulmonary bypass/ECMO. *J Extracorporeal Technol*. 2002;34:144-7.