The Effects of Heparinase 1 and Protamine on Platelet Reactivity

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Background: Protamine is currently the most widely used drug for the reversal of heparin anticoagulation. Heparinase 1 (heparinase) is being evaluated as a possible alternative to protamine for the reversal of heparin anticoagulation. The authors evaluated the effects of equivalent doses of heparinase and protamine on platelet reactivity by measuring agonist-induced P-selectin expression.

Methods: After Institutional Review Board (IRB) approval, informed consent was obtained from 12 healthy volunteers and 8 patients undergoing surgery requiring cardiopulmonary bypass (CPB). Twenty-four ml of blood was obtained from each volunteer; 10 ml of blood was obtained from each patient before the CPB, and another 10 ml was obtained after CPB. Heparin was neutralized using heparinase or protamine. Platelet reactivity was assessed by measuring the expression of P-selectin after stimulation of platelets with increasing concentrations of a thrombin receptor agonist peptide (TRAP). Data were analyzed using analysis of variance. \( P < 0.05 \) was considered significant.

Results: For the healthy volunteers, the activated coagulation times (ACTs) of the heparinized samples returned to baseline values with heparinase (12.5 U/ml) or protamine (32.5 \( \mu g/ml \)). For the 8 patients, the ACTs returned to baseline with heparinase (20 U/ml) or protamine (50 \( \mu g/ml \)). The authors found no difference in the expression of P-selectin in samples neutralized with heparinase, but samples neutralized with protamine showed a significant decrease in the expression of P-selectin when compared with heparinized samples.

Conclusions: At dosages that reverse the anticoagulant effects of heparin, heparinase has minimal effects on platelets, whereas platelet reactivity was markedly inhibited by protamine. (Key words: Blood, platelets: human. Drug: heparinase. Measurement technique: P-selectin expression; flow cytometry. Heparinase. Heparin neutralization. Platelet reactivity. Platelets.)

Despite its known side effects, protamine is currently the most widely used drug for the neutralization of heparin anticoagulation. Heparinase 1 (heparinase) is being evaluated as a possible alternative to protamine for the reversal of heparin anticoagulation. Heparinase is derived from the bacterium Flavobacterium heparinum and neutralizes heparin by enzymatic cleavage of \( \alpha \)-glycosidic linkages at the antithrombin III (AT III) binding site. The mechanism of action of heparinase differs from that of protamine. Protamines are polycationic molecules that neutralize the polyanionic heparin molecules via an acid-base interaction. Protamine and heparinase have been shown to effectively reverse heparin anticoagulation.

The side effects of heparinase have not been extensively evaluated, although Michelson et al. found that heparinase, when compared with protamine, did not produce any significant hemodynamic changes when administered as an intravenous bolus to anesthetized, heparinized dogs. Because the antiplatelet effects of protamine are well documented, we studied the effects of heparinase on platelets in blood from healthy volunteers and from patients undergoing cardiopulmonary bypass.

Materials and Methods

Materials

Bovine lung heparin (heparin sodium, 100 U/ml) was obtained from Upjohn, Kalamazoo, Michigan. Heparinase was obtained from Sigma Chemical Co., St. Louis, Missouri, and was reconstituted as a 1 U/ml solution in 0.15 m NaCl, pH equaled 7.40 (specific activity of 117 U/mg). Murine monoclonal immunoglobulin G (IgG) antibody CD61 (binds to all platelets) conjugated with fluorosothiooctyancar and murine monoclonal IgG antibody CD62 (binds to P-selectin) conjugated with phycocerythrin-streptavidin were obtained from Becton Dickinson Immunocytometric Systems, San Jose, Cali-

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fromia. Thrombin receptor agonist peptide (TRAP) was supplied by Dr. Barry Coller (The Mount Sinai Medical Center, New York, NY). 10mm N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) from Sigma Chemical Co., St. Louis, Missouri (pH, 7.40), was the isotonic buffer used.

Equivalent amounts of the appropriate buffer without reagents were added to the control samples in each experiment so that there were no differences in sample dilution.

Activated Coagulation Time Determinations
The activated coagulation time (ACT), which is a modification of the Lee White whole blood clotting time, was used to determine the endpoints for heparin neutralization. Complete neutralization of heparin anticoagulation was achieved when the ACT returned to the preanticoagulated value. Mixing and incubation of samples were conducted in disposable polypropylene tubes. All ACTs were performed on 2-ml aliquots of whole blood using glass tubes containing 12 mg of celite (Tube 509, International Technidyne, Edison, NJ) in a Hemochron ACT instrument (International Technidyne). After incubation (as will be described), 2 ml of blood was added to a celite-containing tube, and the tube was then placed into a well that had been preheated to 37°C. The tube was continually rotated at 1 rpm, and the clotting time was defined as the interval from the insertion of the tube into the well until clot formation, trapping the magnetic bar and moving it away from a magnetic detector. The ACT values were then recorded.

Heparin Neutralization
After obtaining approval from the Institutional Review Board (IRB), informed consent was obtained from 12 healthy volunteers (Study 1) and 8 patients (Study 2) undergoing surgery requiring cardiopulmonary bypass (CPB).

Study 1. Twelve healthy volunteers were studied: four women and eight men, with a mean age of 29 yr (range, 27–32 yr). From each volunteer, 24 ml of blood was drawn from a peripheral vein using a 21-gauge needle. Fifty units of heparin was added to 20 ml of blood to achieve a heparin concentration of 2.5 U/ml. Baseline ACTs were determined on a 2-ml aliquot of anticoagulated blood and on a 2-ml aliquot of heparinized blood. Heparinase or protamine was added in increasing amounts (heparinase, 3.13 U/ml, 6.25 U/ml, or 12.50 U/ml; protamine, 25 µg/ml or 32.5 µg/ml) to 4-ml aliquots of heparinized blood. Samples were then incubated at 22°C for 2 min, and ACTs were determined on 2-ml aliquots. Platelet analyses, as will be described, were performed on samples of blood before and after complete heparin neutralization.

Study 2. Eight patients were studied: three women and five men, with a mean age of 67 yr (range, 55–82 yr). The ACTs of the nonheparinized blood samples were recorded. Ten milliliters of blood was drawn, from the arterial catheter, from each patient at the following time points: after heparin administration but before the initiation of CPB and after separation from CPB but before protamine administration. ACTs were determined at each time point. The quantity of heparinase or protamine needed for the reversal of heparin anticoagulation was determined by estimating the heparin concentrations. Heparinase (20 U/ml) or protamine (50 µg/ml) was added to 4-ml aliquots of blood drawn at the two time points. Samples were then incubated at 22°C for 2 min, and ACTs were determined on 2-ml aliquots. Platelet analyses, as will be described, were performed on samples of blood before and after complete heparin neutralization.

Assessment of Platelet Reactivity by Flow Cytometry
P-selectin is an adhesion polypeptide that is expressed on the surface of activated platelets. By stimulating platelets with an agonist, the expression of P-selectin reflects the ability of platelets to react. After stimulating platelets, P-selectin expression can be measured using flow cytometry.

Using flow cytometry, all platelets were identified based on light scatter pattern and labeling with the CD61 antibody. The platelet agonist TRAP was used in increasing concentrations, and P-selectin expression was measured using CD62, a murine IgG monoclonal antibody that binds selectively to P-selectin.

Samples for platelet analysis were placed into polypropylene tubes containing one ninth the volume of 3.8% buffered sodium citrate. Samples were then prepared using the technique of Shattil et al. Briefly, 40-ml aliquots of blood were placed into polypropylene tubes. TRAP or buffer was added to each tube to produce final concentrations of TRAP of 0, 0.5, 1.0, or 2.0 nm. The samples were incubated at room temperature for 5 min. Staining with CD61 and CD62 was then performed. After staining, samples were incu-

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Table 1. Activated Coagulation Time (ACT) Values from Healthy Volunteers

<table>
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<tr>
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<th>ACT(s)</th>
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<tr>
<td>Native blood (unheparinized)</td>
<td>127 ± 5</td>
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<tr>
<td>Heparinized (2.5 U/ml)</td>
<td>373 ± 75</td>
</tr>
<tr>
<td>Protamine</td>
<td>25 μg/ml 149 ± 12</td>
</tr>
<tr>
<td>Protamine</td>
<td>32.5 μg/ml 129 ± 10</td>
</tr>
<tr>
<td>Heparinase</td>
<td>3.13 U/ml 286 ± 67</td>
</tr>
<tr>
<td>Heparinase</td>
<td>6.25 U/ml 157 ± 8</td>
</tr>
<tr>
<td>Heparinase</td>
<td>12.5 U/ml 133 ± 6</td>
</tr>
</tbody>
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Values are mean ± SD.

bated at room temperature for 15 min. The samples were then diluted with 500 ml of HEPES buffer. Fifty milliliters of parafomaldehyde fixative solution (Coulter Corp., Miami, FL) was used to stop the reaction. Flow cytometric analyses were performed using the Epics-Profile II Coulter flow cytometer (Coulter, Hialeah, FL).

Statistical Analysis

Analysis of variance (ANOVA) for repeated measures was used to test the effects of heparinase, protamine, or no treatment on TRAP-induced P-selectin expression.

Fig. 1. Effects of heparinase and protamine on P-selectin expression. Heparinized blood from healthy volunteers was neutralized with heparinase or protamine. Data points represent mean ± SD (n = 12). P < 0.01 when compared with P-selectin expression in the heparinized samples for the same concentration of thrombin receptor agonist peptide (TRAP).

Fig. 2. Effects of heparinase and protamine on P-selectin expression. Blood was obtained from patients after the administration of heparin but before the initiation of cardiopulmonary bypass (pre-CPB). Heparin was neutralized with heparinase or protamine. Data points represent mean ± SD (n = 8). P < 0.01 when compared with P-selectin expression in the heparinized samples for the same concentration of thrombin receptor agonist peptide (TRAP).

A post hoc Scheffe’s t test was conducted to check for differences among the groups. P < 0.05 was considered statistically significant.

Results

Study 1

The ACTs of the heparinized samples returned to baseline values with administration of heparinase (12.5 U/ml) or protamine (32.5 μg/ml) but not with smaller amounts of heparinase (3.13 U/ml and 6.25 U/ml) or protamine (25 μg/ml; table 1). We found no difference in the expression of P-selectin in samples neutralized with heparinase, but samples neutralized with protamine showed a significant decrease in the expression of P-selectin at TRAP concentrations of 0.5, 1.0, and 2.0 μM (fig. 1).

Study 2

The ACTs returned to baseline values with heparinase (20 U/ml) or protamine (50 μg/ml). There were no differences in the expression of P-selectin in samples neutralized with heparinase when compared with heparinized samples. Samples neutralized with protamine showed a significant decrease in the expression of P-
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Fig. 3. Effects of heparinase and protamine on P-selectin expression. Blood was obtained from patients after separation from cardiopulmonary bypass but before protamine administration (post-CPB). Heparin was neutralized with heparinase or protamine. Data points represent mean ± SD (n = 8). *P < 0.01 when compared with P-selectin expression in the heparinized samples for the same concentration of thrombin receptor agonist peptide (TRAP).

selectin at TRAP concentrations of 0.5, 1.0, and 2.0 μM (figs. 2 and 3).

Discussion

Protamine is currently the most widely used drug for the neutralization of heparin anticoagulation. Many adverse effects of protamine administration for heparin reversal have been documented, including systemic hypotension, peripheral vasodilatation, pulmonary artery hypertension, thrombocytopenia and leukopenia, decreased cardiac output, and bradycardia.3,11,12 The effects of protamine on platelets also have been well documented. Jaques in 1949 reported significant thrombocytopenia associated with protamine administration.13 More recently, Wakefield et al.14 reported that platelet count decreased by up to 74% after reversal of heparinization with protamine. Qualitative defect of platelet function also has been described. Lindblad et al. studied the effect of protamine sulfate on platelet function and found that protamine inhibits thrombin-induced platelet aggregation.15 Because of the numerous and sometime fatal side effects of protamine, alternative agents are being evaluated.

Heparinase is a possible alternative to protamine. The mechanism of action of heparinase is different from that of protamine. Heparinase neutralizes heparin by enzymatic cleavage of α-glycosidic linkages at the antithrombin III (AT III) binding site.1,2 Michelsen et al.6 recently found that heparinase, when used for the reversal of heparin anticoagulation, did not produce any significant hemodynamic changes.

We evaluated the platelet effects of heparinase. The ACT was used to determine the endpoint for heparin neutralization. Platelet reactivity was evaluated using flow cytometry to measure the expression of P-selectin after stimulating platelets with various concentrations of TRAP. We used this technique to evaluate platelet reactivity because thrombin is considered the most important in vivo agonist for platelets, and by measuring the graded response to TRAP, we can directly evaluate the platelet effects of protamine and heparinase. Another advantage of this technique is that flow cytometry is not affected by platelet count.

We found that heparinase has minimal effects on platelets, whereas protamine markedly inhibits platelet responsiveness. These effects were seen after the neutralization of heparinized samples from healthy volunteers. Blood from patients undergoing surgery requiring CPB showed similar findings, except, as expected, platelet reactivity was depressed immediately after CPB.

To conclude, in vitro heparinase was as effective as protamine in neutralizing heparin anticoagulation but had significantly less effect on platelet reactivity. Heparinase is a promising potential alternative to protamine for the reversal of heparin anticoagulation, although the implications of these in vitro findings will require prospective clinical studies.

References


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