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Platelet Aggregation Following Heparin and Protamine Administration

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The effects of heparin, protamine, and the heparin–protamine complex on the abilities of platelets to aggregate *in vitro* in response to adenosine diphosphate (ADP) and epinephrine were determined. Citrated blood was obtained from normal volunteers and portions were treated with heparin, protamine, and three different ratios of heparin and protamine. The threshold concentrations of ADP and epinephrine required to produce complete platelet aggregation were then determined. Compared with control citrated plasma, the geometric mean of the threshold concentration for ADP in the heparinized sample was decreased twofold, from 1.88 to 0.94 μM; and that for epinephrine more than threefold, from 0.5 to 0.14 μM. In contrast, the threshold concentration for ADP was increased to 3.68 μM in the neutralized and to 2.78 μM in the overneutralized samples and that for epinephrine to 1.62 μM in the neutralized and 1.82 μM in the overneutralized samples. These data indicate that heparin increases the sensitivity of platelets to ADP and epinephrine as determined by platelet aggregation, and protamine added to heparinized blood not only reverses this effect, but decreases platelet sensitivity when it is added in concentrations that neutralize heparin. Additional protamine has no further effect, and protamine alone has no effect on platelet aggregation. (Key words: Blood, anticoagulants; Blood, heparin; Blood, coagulation; Blood, protamine; Blood, platelets.)

PLATELET AGGREGATION is a measure of platelet function that is increasingly employed to assess hemostatic complications of surgery. Many patients evaluated by this test have received heparin, yet reported effects of heparin on platelet function are controversial.[1] Recent articles have reviewed the evidence that heparin may quantitatively or qualitatively alter platelets.[1–3] Much of the controversy can be attributed to the fact that different aspects of platelet function were being observed under variable experimental conditions.

This study has explored the influence of heparin at concentrations utilized during cardiopulmonary bypass on platelet aggregation as usually performed in citrated plasma.[4] Determination of the effect of heparin on the threshold concentration for full response to aggregating agents allowed quantitative comparisons. Furthermore, the effect of the heparin antidote, protamine, on platelet function in concentrations utilized in the neutralization of heparin after bypass has also been explored.

Materials and Methods

After obtaining informed consent, venous blood (250 ml) was collected from 12 normal volunteers in five 50-ml plastic syringes, each containing 5 ml of 3.8 per cent sodium citrate (final concentration of citrate, 0.013 M). Heparin§ was added to four aliquots to attain a final concentration of 3.0 unit/ml. The contents of the fifth syringe served as a control. Protamine, 30 μg/ml, was added to a sixth syringe of blood obtained from six of the volunteers. After mixing, the exact concentration of protamine¶ necessary for neutralization of the heparin in those samples that had been heparinized was determined by measuring thrombin times.[5]

Determination of the Neutralizing Concentration of Protamine

Human thrombin** was diluted with 20 ml buffer (0.02 M barbital buffer containing 0.15 M NaCl, *p*H = 7.60) sufficient to produce a thrombin clotting time in normal control plasma of approximately 20 seconds. To determine the dose of protamine needed for neutralization of heparin, protamine was added in 5-μg increments from 20 to 50 μg to 1.0-ml samples of blood from a heparinized syringe. The samples were then centrifuged as in the preparation

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§ Riker Laboratories Northridge, California 91324, derived from porcine intestinal mucosa, 1,000 μ/ml.

¶ Eli Lilly and Co., Indianapolis, Indiana 46206, 10 mg/ml.

** Fibrindex, Ortho Diagnostics Inc., Raritan, New Jersey 08869, 50 units/ampule.

TABLE 1. Representative Thrombin Time Calculation

Heparin, Units/ml	Protamine, μg/ml Whole Blood	Thrombin Time, Seconds
0	0	20.6
3.0	0	>100
3.0	20	>40.0
3.0	25	40
3.0	30	33.7
3.0	35	22.8
3.0	40	21.9*
3.0	45	22.7
3.0	50	22.8

* 40 μg protamine per ml was used as the basis for calculating the exact neutralizing dose, since that dose produced the shortest thrombin time, virtually equal to the control. Thus, 36 (90 per cent), 40 (100 per cent), and 60 (150 per cent) μg protamine per ml were required, respectively, for the underneutralized, exactly neutralized, and overneutralized samples. The calculated doses of protamine, 1,800, 2,000, and 3,000 μg, were then added to the 50-ml volumes to be underneutralized, exactly neutralized, and overneutralized, respectively.

of platelet-poor plasma (see below). To 0.2 ml aliquots of these plasma samples, 0.2 ml of the diluted thrombin were added and thrombin times determined utilizing an automated device.†† The amount of protamine present in the sample with the shortest thrombin time was used as the basis for calculating the neutralizing dose for heparin in blood samples in three 50-ml syringes. To the first, which was to be underneutralized, 90 per cent of the calculated concentration was added. To the second, which was to be exactly neutralized, 100 per cent of the calculated concentration was added. To the third, which was to be overneutralized, 150 per cent of the calculated concentration was added. Table 1 illustrates this calculation for a typical blood sample.

Aggregating Agents

Adenosine diphosphate‡‡ was stored frozen at a concentration of 5×10^{-4} M in 0.1 M sodium phosphate buffer, pH = 6.80. A stock refrigerated solution of l-epinephrine,‡‡ 2.5×10^{-4} M in HCl, neutralized to pH 7.00 with sodium hydroxide, was used.

Preparation of Platelet-rich and Platelet-poor Plasma

Blood samples were centrifuged at $17 \times g$ for 10 minutes; the resultant platelet-rich plasma had a platelet count range of 260,000–418,000/μl (mean = 373,000 ± 23,000 SEM). The remaining blood was then centrifuged at $12,000 \times g$ for 4 minutes to produce platelet-poor plasma with a platelet count consistently less than 1,000/μl. All operations were performed in plastic tubes at 23 C.

Platelet Aggregation

Platelet aggregation was studied in an aggregometer§§ in which light transmittance through samples of plasma is measured. The percentage transmittance through platelet-rich plasma is set at zero and that through platelet-poor plasma is set at 100 per cent. When platelet aggregating agents are added to the platelet-rich plasma, the percentage transmittance will increase in proportion to the amount of platelets that aggregate and settle out of suspension.

Platelet-rich plasma, 0.5 ml, was stirred at 1,200 rpm at 37 C in siliconized cuvettes, 8 mm in diameter, with the use of a plastic bar, 1.0 × 0.5 mm. The threshold concentrations of ADP and epinephrine, which are the minimal concentrations of the two aggregating agents necessary to produce secondary aggregation, were then determined.[6] For 15 normal individuals these were found to be transmittances of 79 ± 4.3 (SD) per cent for ADP and 82 ± 4.2 per cent for epinephrine. Thus, the minumum values for complete responses two standard deviations below the mean were 71 per cent for ADP and 74 per cent for epinephrine.[7]

Statistical Methods

Groups were compared by an analysis of variance using the log doses of aggregating agents since their distribution is lognormal. Analysis was performed on the logarithms of individual values.

Results

Effect of Heparin

The geometric mean concentration of ADP necessary to produce maximum transmittance (secondary aggregation) was decreased twofold from 1.88 to 0.94 μM following heparin (table 2) The threshold concentration of epinephrine was decreased more than threefold, from 0.50 to 0.14 μM. Both differences were significantly greater than control ($P < 0.05$).

Effect of Protamine Neutralization

Analysis of variance failed to show a significant difference in responses of the aggregation of platelets to ADP between control and underneutralized samples or between control and protamine-only

†† Fibrometer, Bioquest, Baltimore, Maryland.

‡‡ Sigma Chemicals, St. Louis, Missouri.

§§ Chrono-Log Corp, Havertown, Pennsylvania 19083.

TABLE 2. Minimal Concentrations of ADP and Epinephrine Necessary to Produce Secondary Aggregation*

Sample	Control	Heparin	Heparin:Protamine Ratio			Protamine
			1.0:0.9	1.0:1.0	1.0:1.5	
ADP (in μM)	1.88	0.94†	2.47	3.68†	2.78†	2.77
Range (95 per cent)	1.0–5.0	0.5–2.0	0.5–10.0	1.0–10.0	1.0–10.0	1.0–5.0
Epinephrine (in μM)	0.50	0.14†	0.66	1.62†	1.82†	0.50
Range (95 per cent)	0.01–1.0	0.025–0.25	0.25–5.0	0.25–10.0	0.25–25.0	0.1–5.0

* The more sensitive platelets require a lower concentration of aggregating agent. Thus, heparin enhances platelet aggregation and the heparin–protamine complexes impair platelet function.

† Mean differs significantly from control mean, $P < 0.05$, using two-dimensional analysis of variance.

samples. However, with both neutralizing and overneutralizing doses of protamine the amounts of ADP needed to produce secondary aggregation were significantly greater than control ($P < 0.05$).

Similarly, following heparin neutralization with protamine, the amounts of epinephrine needed to produce secondary aggregation were greater in both neutralized and overneutralized samples, resulting in a statistically significant difference ($P < .05$). However, again there was no statistically significant difference in the responses of platelets to epinephrine between control and underneutralized samples or between control and protamine-only samples.

Discussion

O'Brien has pointed out the difficulty in analyzing the effect of heparin on platelet function by observing: "To what should the effect be compared?" Native blood cannot be used for comparison because it will clot. Comparison with blood anticoagulated with citrate will invariably produce an artifact, since citrate decreases ionized calcium, which modulates platelet function.[2] In addition, there are more than 30 different heparin preparations available, differing in molecular weights and cation compositions, and these may have variable effects.[8] While it is conceivable that platelets with an abnormal *in-vitro* response may behave normally *in vivo*, a normal platelet count is not an absolute indication of normal platelet function.[9]

Our previous work challenged the belief that protamine has a clinically important effect on the coagulation mechanism.[4] Subsequently, it was suggested that the clinical observation of increased bleeding following excess protamine might be due to its effects on platelets.[10,11] The results of this *in-vitro* study indicate that doses of protamine that result in exact heparin neutralization adversely affect platelet aggregation in response to ADP or epinephrine (table 2). Since protamine alone has no effect on platelet aggregation, decreased sensitivity to aggregating agents must be due to the heparin–protamine complex.

Platelet function is depressed following cardiopulmonary bypass.[12–13] This fact, combined with a failure to show a further decrease in sensitivity between the neutralized and overneutralized doses of protamine, suggests that additional protamine may have no adverse effect on the contribution of platelets to effective surgical hemostasis. However, when platelets are administered in the form of either homologous platelet concentrates or fresh autologous blood, the function of these platelets should not be depressed. The demonstrated decreases in platelet sensitivity apparently due to the heparin–protamine complex, combined with reports of significantly increased bleeding following higher doses of protamine,[10] suggest that more care must be taken in administering protamine than we had previously advocated. Recently developed tests that permit more accurate determination of the extent of heparinization and its neutralization by protamine make the determination possible in a clinical setting.[4,14]

As present efforts directed at developing means to protect platelets during extracorporeal circulation continue, the effect of heparin–protamine on platelet sensitivity to aggregating agents may become more important. Both platelet-compatible surfaces and protective additives are being evaluated as means of preserving platelets and their function during bypass.[13,15] The conclusion that the heparin–protamine complex decreases platelet sensitivity to aggregating agents twofold would be even more meaningful at that time.

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