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Anticoagulant Effects of Protamine Sulfate in a Canine Model[1,2]

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Protamine sulfate is considered a weak anticoagulant, yet little is known concerning the mechanism of this effect or its relation to prior heparin exposure. This investigation defined the influence of increasing doses of protamine, with and without prior heparin anticoagulation, on the activated clotting time (ACT), thrombin clotting time (TCT), prothrombin time (PT), partial thromboplastin time (PTT), fibrinogen level, platelet count, and platelet aggregation to ADP in dogs (n = 8). Four doses of intravenous protamine sulfate (1.5, 3.0, 6.0, and 15.0 mg/kg) were studied in each animal, with at least 5 days between individual studies. Four dogs received heparin, 150 IU/kg 10 min prior to protamine sulfate administration, and four dogs received protamine sulfate alone. Protamine sulfate caused anticoagulation, both in the presence and absence of heparin, with significant changes occurring in the ACT, PTT, platelet count, and platelet aggregation. Relevant changes did not occur in the TCT, PT, or fibrinogen levels. Platelet effects were capable of causing bleeding with standard or excess use of protamine sulfate, especially if platelet numbers were already decreased, as might occur in surgical procedures where thrombocytopenia commonly accompanies major blood loss and replacement. The ACT, reflecting both the coagulation cascade and platelet function, was the test most profoundly affected by protamine overdosage, and therefore may be misleading as a measure of protamine reversal of heparin. The TCT, which is sensitive to heparin anticoagulation but not protamine-induced anticoagulation, should be more accurate in differentiating inadequate heparin reversal from the effects of excess protamine.

INTRODUCTION

Postoperative bleeding continues to be a serious complication of cardiovascular surgery. Protamine sulfate is commonly used to reverse heparin anticoagulation. This substance has been shown to have anticoagulant properties when used in large doses [1, 2] and is known to cause platelet aggregation and profound decreases in platelet counts [3, 4]. Compounding these events is the fact that protamine reversal of heparin may also cause adverse hemodynamic effects [4].

The activated clotting time (ACT) is often used intraoperatively to assess the effect of initial heparin dose and to titrate it's later reversal [5, 6]. This becomes important because of the variable response to heparin resulting in potentially inadequate anticoagulation if a fixed heparin dose is based only on body weight, and if inadequate or excessive protamine administration at the time of heparin reversal is to be avoided. The ACT responds in a linear fashion to increasing heparin dosage and correlates well with the more cumbersome Lee-White clotting times [7]. Many authors have alluded to the phenomenon of heparin rebound, with a slow return of heparin activity after apparent complete protamine reversal, as a limitation to titrating protamine dosage with intraoperative clotting times [8, 9]. This rebound phenomenon may be due to metabolism of protamine by serum proteases with release of heparin from heparin-protamine complexes [10]. Whatever the cause, it has been suggested that excess protamine be administered for heparin reversal in order to prevent a later anticoagulated state and possible bleeding [8,

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[2] Agents used in this study included beef lung heparin, Upjohn Co. (Kalamazoo, MI), and protamine sulfate, Eli Lilly and Co. (Indianapolis, IN).

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9]. Furthermore, it has been suggested that a moderate excess of protamine will not cause a clinically significant state of anticoagulation [11].

The purpose of this investigation was to better define the anticoagulant action of protamine sulfate, both in the presence and absence of prior heparin administration, with specific attention to activated clotting times and platelets, as well as other standard parameters of coagulation.

METHODS

Eight awake adult female mongrel dogs weighing 20 to 30 kg received four different doses of protamine sulfate (1.5, 3.0, 6.0, and 15.0 mg/kg) given intravenously over a 5-min time period with at least a 5-day interval between studies of different doses in each subject. Four animals (Group I) were anticoagulated with intravenous sodium heparin, 150 IU/kg, given as a bolus 10 min prior to protamine administration. Four other animals (Group II) did not receive heparin prior to protamine administration. Blood specimens were obtained from a superficial limb vein prior to drug administration in all animals, 5 min after heparin administration in animals that received heparin, and 5 minutes after completion of protamine administration in all animals (Fig. 1).

Blood for coagulation testing and platelet aggregation studies was drawn into evacuated tubes containing buffered citrate (Becton–Dickinson, Rutherford, NJ). Tubes containing EDTA were used to collect blood for platelet counts. Platelin Plus Activator, composed of rabbit brain phospholipids and Celite, (Organon Teknika Co., Morris Plains, NJ), was used for the ACT assays. Data-Fi thrombin reagent was used for the thrombin clotting time (TCT) and fibrinogen determinations, with actin-activated cephaloplastin reagent used for measuring activated partial thromboplastin time (PTT), and thromboplastin-C rabbit brain thromboplastin with calcium reagent used for determining prothrombin times (PT), (all reagents from American Hospital Supply del Caribe, Inc., Aquada, Puerto Rico). The sodium salt of ADP, grade I/95–99%, (Sigma, St. Louis, MO) was used as the platelet aggregant. A TRIMED ACTester Model PA1000 (Huntington Beach, CA) was used for determinations of whole blood activated clotting times. The PT, PTT, TCT, and fibrinogen assays were performed with a fibrometer (Model 60415, Becton–Dickinson, Cockeysville, MD) and platelet aggregation studies were done using a dual-sample aggregation meter (Model DP-247-E, Sienco, Morrison, Co).

The ACT was determined immediately after blood drawing by adding 0.2 ml of whole blood to 0.1 ml of Platelin Plus and then measuring the time of clot formation with the ACTester. For the other studies citrated blood was centrifuged at 800–1000 rpm for 5 min and the plasma was decanted as platelet rich plasma (PRP). These samples were then centrifuged at 1800–2000 rpm for an additional 10 min and the plasma was decanted as platelet poor plasma (PPP). The PPP was utilized for determination of the TCT, PT, PTT, and fibrinogen levels with the fibrometer. Platelet counts by phase-contrast microscopy were done on the PRP samples, so that platelet numbers could be standardized for aggregation testing. Autologous PPP was used to dilute PRP to a platelet count of 200,000 or to the lowest platelet count in each animal's baseline, postheparin, or postprotamine PRP samples, if any of these samples had counts less than 200,000. Platelet aggregation to ADP, at a concentration of 1 μg/ml, was determined with the aggregometer calibrated such that 5% corresponded to untreated PRP and 95% corresponded to PPP. The maximum aggregation level in response to ADP was used as the index of platelet function in this study. Whole blood platelet counts were determined on the EDTA blood samples by dilution, red cell lysis, and phase-contrast microscopy.

Data were assessed using actual test values and also as the ratio or percentage of post-treatment values compared to baseline

values for each animal. A nonparametric median test was used to compare post-treatment values to the baseline values. Data in this work are expressed as the $\bar{x} \pm 1$ SD. Animal care complied with the *Principles of Laboratory Animal Care* and the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 80-23, Revised 1976).

RESULTS

ACT baseline values in Group I ranged from 69 to 108 sec with a mean of 94 ± 10 sec ($n = 15$). After administering 150 IU/kg of heparin, the ACT ranged from 225 to more than 1000 sec (1000 being the maximum value measured on the ACTester). This corresponded to an increase of 2.3 to greater than 14.5 times the baseline value (3.7 ± 4.5). TCT increased 7.5- to 20-fold after heparin administration. Increases in PTT were 1.6- to 5.2-fold, and increases in PT were 0.9 to 1.7 times baseline values. Heparin administration did not cause statistically significant changes in fibrinogen (range 80 to 117% of baseline) or platelet counts (68 to 119% of baseline). The platelet aggregation response to heparin varied widely (61 to 600% of baseline). Some animals exhibited increased aggregation and some had decreased platelet aggregation after heparin, resulting in no overall significant differences. In Group II the ACT baseline values ranged from 77 to 135 sec (101 ± 15).

Coagulation tests in this investigation were expressed in absolute terms or in comparison to baseline values (Tables 1 and 2). Normalized values for the ACT, TCT, PT, and PTT tests in this study were defined as a ratio to baseline, whereas normalized values for fibrinogen level, platelet count, and platelet aggregation were expressed as a percentage of the baseline (Figs. 2 and 3).

A protamine reversal dose of 1.5 mg/kg, corresponding to 1 mg protamine for each 100 units of heparin, returned coagulation tests altered by heparin to baseline values. The 1.5 mg/kg dose of protamine did not produce significant anticoagulation. Protamine administration in increasing doses resulted in progressive prolongation of the ACT and PTT. Progressively larger decreases in platelet counts also occurred with increasing protamine dose. Diminutions in platelet counts became dramatic with protamine

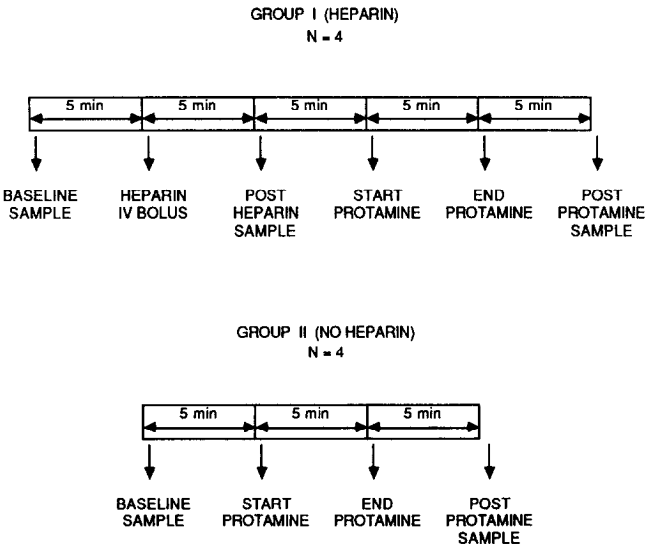

FIG. 1. Study design.

TABLE 1

EFFECTS OF INCREASING DOSES OF PROTAMINE ON COAGULATION IN GROUP I ANIMALS WITH PRIOR HEPARIN (150 IU/kg)

Protamine dose (mg/kg)	1.5	3.0	6.0	15.0
		Actual		
ACT (sec)	99.0 ± 27.0	109.0 ± 5.0*	171.0 ± 47.0*	861.0 ± 278.0*
TCT (sec)	7.1 ± 2.2	6.7 ± 0.7	6.3 ± 1.2	6.4 ± 1.0
PT (sec)	7.1 ± 0.6	6.9 ± 0.6	7.1 ± 0.5	7.7 ± 1.2
PTT (sec)	12.6 ± 1.4	11.4 ± 1.5	13.3 ± 1.0	33.4 ± 28.8*
Fibrinogen (mg/dl)	244.0 ± 83.0	310.0 ± 173.0	367.0 ± 214.0	267.0 ± 217.0
Platelet count (number/mm^3)	159.0 ± 81.0	162.0 ± 107.0	159.0 ± 115.0	44.0 ± 47.0*
Platelet aggregation (max %)	27.0 ± 6.0	24.0 ± 10.0	13.0 ± 2.0*	N/A[a]
		Normalized		
ACT	1.1 ± 0.3	1.2 ± 0.1*	1.7 ± 0.5*	10.1 ± 4.3*
TCT	1.1 ± 0.4	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1
PT	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.2
PTT	1.2 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	3.2 ± 2.8*
Fibrinogen	94.0 ± 15.0%	89.0 ± 22.0%	100.0 ± 8.0%	70.0 ± 19.0%
Platelet count	45.0 ± 15.0%	41.0 ± 7.0%	35.0 ± 17.0%	10.0 ± 9.0%*
Platelet aggregation	99.0 ± 67.0%	59.0 ± 19.0%	45.0 ± 17.0%*	N/A[a]

Note. Data are expressed as means ± standard deviations for actual values and data normalized to baseline (preheparin) values.

[a] Low platelet count precluded aggregation testing.

* $P < 0.05$.

overdosage, and platelet clumping was observed by phase microscopy after protamine administration. Similarly, a progressive drop in platelet aggregation occurred in response to ADP with increasing doses of protamine. Aggregation studies could not be done with the 15.0 mg/kg protamine dose because the platelet count was too low. TCT, PT, and fibrinogen levels did not reflect the anticoagulant properties of protamine. The aforenoted changes in the coagulation system occurring with protamine overdosage were similar in both heparinized and nonheparinized animals.

DISCUSSION

Significant anticoagulant properties of protamine sulfate were documented in this study, with demonstrated effects noted on both platelet function and the coagulation cascade. The drop in platelet count associated with protamine sulfate reversal of heparin has been well documented previously [2, 4]. This phenomenon is most likely secondary to the platelet aggregatory properties of protamine sulfate [3]. Platelets are known to be sequestered in the lung after protamine administration [12]. Protamine-induced platelet aggregation as a cause of the thrombocytopenia was consistent with the platelet aggregates seen on phase microscopy in our samples. Protamine-induced platelet aggregation may be due to its direct effect on platelet membranes or it could be mediated by recognized aggregants such as thrombin. In this regard, protamine is known to interfere with thrombin neutralization by antithrombin III and also thrombin binding to thrombomodulin [13, 14]. Blocking these neutralizers of thrombin's procoagulant action may make more thrombin available for binding to platelet receptors, thus resulting in platelet aggregation.

In this study, diminutions in platelet counts were progressively greater with increasing protamine doses. The impressive thrombocytopenia occurring with protamine overdosage under these circumstances could result in bleeding. A drop in platelet count to 20 to 30% of baseline with a protamine reversal dose of 1.5 mg/100 units of heparin has been seen in earlier studies from our laboratory [15, 16], and we have noted a drop to 73% of baseline in humans using this same reversal dose [17]. It is possible that human platelets are less sensitive to protamine or that medications inhibiting platelet aggregation in patients are responsible for the lesser degree of protamine-induced thrombocytopenia occurring in humans.

Platelet aggregation to ADP was progressively inhibited by increasing doses of protamine in this study. In a previous experiment from our laboratory using platelets exposed to exogenous protamine, inhibition of thrombin-induced platelet aggregation occurred, but ADP-induced platelet aggregation was not inhibited [18]. Since protamine is a platelet aggregant, it is possible that the apparent drop in platelet aggregation is a result of selecting out the more responsive platelets by protamine, rather than actual protamine inhibition of platelet aggregation. In any case, the end result of protamine administration is a lowered number of platelets and a lessened response to platelet aggregants.

The coagulation cascade also appears to be affected by protamine sulfate administration. Protamine's greater effect on the PTT than the PT suggests that enzymes or cofactors of the intrinsic pathway are more sensitive to this substance. Failure of protamine to affect the TCT suggests that inhibition of thrombin-catalyzed conversion of fibrinogen does not contribute significantly to protamine's anticoagulant effect. Protamine is a

TABLE 2

EFFECTS OF INCREASING DOSES OF PROTAMINE ON COAGULATION IN GROUP II ANIMALS WITHOUT PRIOR HEPARIN

Protamine dose (mg/kg)	1.5	3.0	6.0	15.0
		Actual		
ACT (sec)	102.0 ± 10.0	134.0 ± 18.0*	313.0 ± 215.0*	1000.0 ± 0.0*
TCT (sec)	6.8 ± 1.5	6.6 ± 0.5	6.5 ± 1.5	6.2 ± 1.0
PT (sec)	6.9 ± 0.5	7.1 ± 0.6	7.4 ± 1.0	8.2 ± 0.4*
PTT (sec)	11.4 ± 1.2	11.1 ± 1.6	15.7 ± 5.0*	29.9 ± 5.4*
Fibrinogen (mg/dl)	460.0 ± 240.0	422.0 ± 244.0	340.0 ± 139.0	316.0 ± 170.0
Platelet count (number/mm^3)	328.0 ± 161.0	140.0 ± 54.0*	56.0 ± 32.0*	55.0 ± 48.0*
Platelet aggregation (max %)	38.0 ± 21.0	35.0 ± 17.0*	28.0 ± 8.0*	N/A[a]
		Normalized		
ACT	1.0 ± 0.1	1.4 ± 0.3*	3.2 ± 2.1*	9.7 ± 2.4*
TCT	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1
PT	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.3 ± 0.1*
PTT	1.0 ± 0.1	1.1 ± 0.1	1.5 ± 0.5*	2.7 ± 0.3*
Fibrinogen	107.0 ± 18.0%	98.0 ± 6.0%	96.0 ± 4.0%	85.0 ± 14.0%
Platelet count	88.0 ± 5.0%	46.0 ± 25.0%*	14.0 ± 7.0%*	13.0 ± 10.0%*
Platelet aggregation	63.0 ± 26.0%	51.0 ± 28.0%*	37.0 ± 1.0%*	N/A[a]

Note. Data are expressed as means ± standard deviations for actual values and data normalized to baseline (pre-protamine) values.

[a] Low platelet count precluded aggregation testing.

* $P < 0.05$.

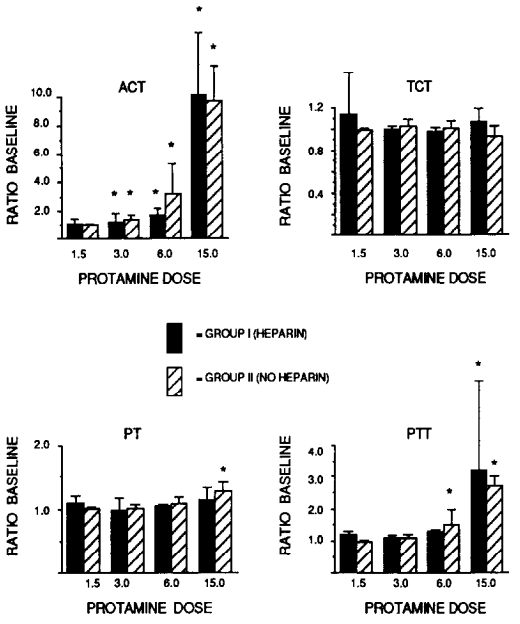

FIG. 2. Effects of increasing protamine dose on activated clotting time (ACT), thrombin clotting time (TCT), prothrombin time (PT), and partial thromboplastin time (PTT). Error bars represent standard deviations. $^*P < 0.05$.

substrate for thrombin, and has been shown to block thrombin conversion of fibrinogen in an *in vitro* assay [19]. In the present study this property did not appear to be an important factor in the protamine-associated state of anticoagulation. The results of this investigation imply that inhibition of the protease factors XII, XI, and IX or the protease cofactor factor VIII contributes to the anticoagulant effects of protamine. Inhibition of factor XI by protamine has been demonstrated [20].

ACT appears to be the clotting assay most sensitive to the anticoagulant properties of protamine sulfate. Since ACT is a whole blood clotting study, it's sensitivity could be explained by protamine's dual effect on both platelets and the coagulation cascade. Although the ACT may best reveal the overall state of anticoagulation, it may be misleading when used as a guide for protamine sulfate reversal of heparin. Considerable error may exist if a prolonged ACT following protamine sulfate reversal of heparin is interpreted as always indicating incomplete reversal. Indeed, prolonged ACT values can reflect other causes of anticoagulation including excess protamine. Administration of additional protamine under such circumstances may only heighten the degree of existing anticoagulation. Since the TCT is a sensitive and accurate indicator of heparin activity and is not significantly affected by protamine excess, it should be more accurate in differentiating inadequate heparin reversal from protamine overdose. The TCT is affected only by fibrinogen concentration and thrombin inhibitors. Perhaps a modified activated whole blood clotting time with thrombin as the activating agent would be more useful for intraoperative monitoring of heparin activity and it's reversal with protamine.

Protamine sulfate acts as an anticoagulant with effects on platelets and the intrinsic coagulation pathway. These effects should not lead to significant anticoagulation with standard doses of protamine if the coagulation system is normal. However, the antiplatelet

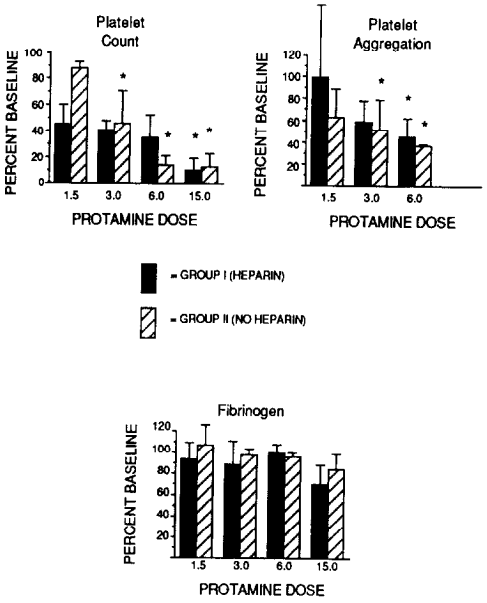

FIG. 3. Effects of increasing protamine dose on platelet count, platelet aggregation to ADP, and fibrinogen. Error bars represent standard deviations. $^*P < 0.05$.

properties of protamine sulfate may become more important in the face of prexisting thrombocytopenia or altered platelet function. The ACT reflects the combined anticoagulant and antiplatelet effects of protamine. Thus, a prolonged ACT after protamine reversal of heparin may be misleading if it is routinely interpreted as indicating inadequate reversal. The TCT is not affected by large excesses of protamine and is therefore considered a more reliable indicator of residual heparin activity after protamine administration.

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