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PROTAMINE REVERSAL OF ANTICOAGULATION ACHIEVED WITH A LOW MOLECULAR WEIGHT HEPARIN. THE EFFECTS ON EICOSANOIDS, CLOTTING AND COMPLEMENT FACTORS

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ABSTRACT

Hemodynamic and hematologic effects of protamine reversal of low molecular weight heparin (LMWH) anticoagulation with and without protamine pretreatment, as well as reversal of anticoagulation with unfractionated standard heparin (SH), were studied in canine subjects. Protamine reversal caused less severe thrombocytopenia in the two LMWH groups compared to SH animals, while neutropenia occurred equally in all groups. C1-esterase inhibitor levels were minimally increased, whereas C3 levels and leucotriene levels were unaltered. TxB_2 and 6-keto-$PGF_{1\alpha}$ increased during protamine reversal of LMWH anticoagulation. TCT and APTT were affected less with LMWH than SH anticoagulation. Anti-Xa levels increased with anticoagulation in all animals, but protamine did not reverse the elevated anti-Xa levels in LMWH anticoagulated dogs to the same degree as occurred with SH anticoagulation. TCT, APTT and bleeding times were normalized by protamine in all animals. Protamine reversal of LMWH anticoagulation with or without protamine pretreatment did not reveal any clear differences in eicosanoids or complement factors compared to SH anticoagulation, although differences in anti-Xa activity clearly separated these two heparins.

INTRODUCTION

Protamine sulfate reverses heparin anticoagulation by dissociation of heparin-antithrombin III complexes and the formation of protamine-heparin complexes. Protamine sulfate may also cause systemic hypotension, bradycardia, pulmonary artery hypertension, thrombocytopenia, and neutropenia,

Key Words: Protamine, heparin, low molecular weight heparin, complement, eicosanoids.

as well as allergic and anaphylactic-like reactions during heparin reversal (1,2). The exact mechanisms by which these adverse side effects occur have not been clearly defined.

In contrast to unfractionated standard heparin (SH), the antithrombotic potential of low molecular weight heparin (LMWH) depends on inhibition of factor Xa, and has a lesser effect on factor IIa. In addition, LMWH appears to have less of an effect on platelets. Pharmacokinetically, anticoagulant levels of LMWH remain detectable for longer periods of time than with SH. Other potential advantages of LMWH over SH include fewer disturbances of liver function and lipolysis, as well as a lesser demineralizing effect on the skeleton (3).

Recently it was demonstrated that pretreatment with a small dose of protamine before subsequent reversal of SH anticoagulation reduced the adverse hemodynamic and hematologic side effects usually accompanying such reversal. The hemodynamic effects of protamine reversal of anticoagulation achieved with LMWH as well as with SH have recently been assessed with and without protamine pretreatment (1,2). The current study investigates changes in eicosanoids, clotting, and complement factors occurring with reversal of SH and LMWH anticoagulation in an experimental canine model in which a prosthetic graft was placed in the infrarenal aortic position.

MATERIALS AND METHODS

Eighteen mongrel dogs of both sexes were prepared for study: Group I (n=6) received LMWH following saline pretreatment, Group II (n=6) received LMWH following protamine pretreatment, and Group III (n=6) received SH following saline pretreatment. All animals were anesthetized with sodium pentobarbital (30 mg/kg) and mechnically ventilated. Hydration was maintained by administration of lactated ringers solution, as a 20 ml/kg bolus followed by a 10 ml/kg/hour infusion. An inferior vena cava catheter was placed transfemorally for blood sampling. Drug administration was performed through a separate peripheral intravenous cannula. Systemic arterial blood pressure measurements were made using a catheter in the femoral artery, while pulmonary artery pressures and cardiac output determinations were accomplished with a Swan Ganz catheter threaded from the femoral vein into the pulmonary artery. The infrarenal aorta was exposed through a midline abdominal incision for placement of a 6 mm ID Dacron double velour aortic interposition graft, 5 to 7 cm length.

Protamine or saline pretreatment was randomly administered to study subjects in a blinded fashion. Protamine pretreatment consisted of 2.25 mg/kg protamine sulfate (Upjohn Inc., Kalamazoo, MI, USA) divided into three equal doses (0.75 mg/kg) given at 2 minute intervals prior to heparin administration. An equal volume of 0.9N NaCl solution was administered in the saline pretreatment groups. Anticoagulation was then achieved with LMWH or SH. Heparin was administered 3 minutes following completion of pretreatment, and 3 minutes later the aorta was clamped.

The LMWH fragment used in this study was obtained by partial degradation of porcine intestinal mucosal heparin with nitrous acid and isolated by ethanol precipitation. LMWH antiXa activity was 4.1 times its APTT activity. This LMWH had a medium molecular weight of 4,000-5,000 (Kabi 2165, Kabi, AB, Sweden). LMWH was given in a dose of 150 U antiXa/kg. SH of beef lung origin was administered in a dose of 150 IU/kg (UpJohn Inc., Kalamazoo, MI, USA).

Five minutes after completing the insertion of the aortic prosthesis intravenous protamine sulfate (1.5 mg/kg) was administered over a 10 second time period. Blood samples for biochemical and hematological studies were obtained at baseline, 2 minutes after protamine pretreatment, 2 minutes after heparin anticoagulation, as well as immediately before and 1, 3 and 10 minutes after protamine reversal.

Platelet and white blood cell counts were performed using standard techniques. α_2-macroglobulin was assayed semiquantitatively by titration of plasma samples with porcine trypsin using N-Benzoyl-DL-Arginine-p-Nitro-aniline (4) (BapNA, Sigma Chemical Co., St. Louis, MO, USA). C-1-esterase inhibitor (Chromogenic substrate S-2302, Kabi AB, Sweden) was determined according to the technique of Gallimore and Friberger (5). Antithrombin III analysis (Chromogenic substrate S-2238, Kabi AB, Sweden) was performed as described by Abildgaard et al (6). Heparin activity was assessed by anti-Xa analysis (Chromogenic substrate S-2222, Kabi AB, Sweden) as reported by Teien et al. (7). All chromogenic substrate assays were endpoint assays. Standard curves were made from pooled baseline plasma. Beef heparin related to 3rd Int. Std. was used. C3 was determined by electroimmunophoresis (8).

TABLE 1.
Platelet and White Blood Cell Counts Accompanying Protamine Reversal of Anticoagulation

	Group I LMWH, Saline Pretreatment	Group II LMWH, Protamine Pretreatment	Group III SH, Saline Pretreatment
	Platelet Count (10^3/mL)		
Baseline	253±33	210±30	231±49
After pretreatment	266±35	170±24**	228±31
After heparinization	248±30	159±19*	258±44
Before reversal (rev.)	271±40	200±25	259±43
1 minute after rev.	176±39***	103±10*	67±21***
3 minutes after rev.	111±14***	103±29**	96±20**
10 minutes after rev.	131±23***	100±21**	86±9**
	White Blood Cell Count (10^3/mL)		
Baseline	8.8±1.4	6.3±1.4	7.5±0.5
After pretreatment	9.0±1.6	6.4±1.6	6.1±0.3
After heparinization	7.2±1.3	7.7±2.1	7.2±1.5
Before reversal (rev.)	7.5±1.1	8.5±1.6	7.4±1.3
1 minute after rev.	3.6±0.6*	3.3±0.5*	3.3±0.6*
3 minutes after rev.	4.6±0.8	6.0±0.7	5.8±1.0
10 minutes after rev.	7.7±1.1	7.4±1.1	8.6±1.4

Data expressed as x ± SEM; n=6 in each group; Intragroup differences *p<0.05, **p<0.01, ***p<0.001.

Eicosanoid activity was quantitated in plasma after extraction of proteins and free fatty acids. Metabolites of prostacyclin (6-keto-$PGF_{1\alpha}$) and thromboxane (TxB_2) were measured by radioimmunoassay (New England Nuclear, detection limit 3.8 pg and 0.5 pg, respectively). Cross-reactivity to these related eicosanoids was low. Leucotrienes C_4 and B_4 were also measured using radioimmunoassay technique (New England Nuclear kit, detection limit 3.3

pg and 1.5 pg, respectively). Statistical analyses included student's t-test for intragroup paired data and Wilcoxon rank sum test for intergroup data comparisons.

RESULTS

Protamine reversal of anticoagulation produced reductions in platelet counts (-59%, -50%, -74%) and white blood cell counts (-52%, -61%, -55%) in groups I, II and III, respectively (Table 1). Depressed platelet counts persisted during the study, but white blood cell counts generally normalized within 10 minutes. Protamine pretreatment in and of itself (Group II) produced a slight reduction (-19%) in platelet count but no effect on the white blood cell count.

Antithrombin III and α_2-macroglobulin levels were essentially unchanged following protamine reversal in groups I and II (Table 2). Anti-Xa levels were greater than 1.0 heparin U/ml after heparinization in all 3 groups. Anti-Xa activity returned to baseline in group III animals but remained elevated in both groups of animals anticoagulated with LMWH (Table 3).

C-1-esterase inhibitor levels were not statistically changed after protamine reversal in any group, but did tend to increase at 1 and 3 minutes after reversal. C3 activity did not change significantly in any of the groups (Table 4).

6-keto-$PGF_{1\alpha}$ levels increased during aortic clamping (+123%) and increased further after reversal in group I. There was no increase in 6-keto-$PGF_{1\alpha}$ during clamping in group II although there was an increase with protamine reversal of heparin (Table 5). Protamine reversal caused increased activities of TXB_2 in group I compared to group II (Table 5). LTB_4 and LTC_4 levels were not significantly altered (Table 6).

Different assays regarding anticoagulation were used in addition to XaI measurements (Table 7). SH had a more pronounced effect on TCT and APTT then LMWH. LMWH with protamine pretreatment (Group II) affected APTT and TCT less than with saline pretreatment (Group I), although the same effect on factor XaI was observed (Table 3).

TABLE 2.
Antithrombin III and α_2-Macroglobulin Levels
During Anticoagulation With Low Molecular Weight Heparin and Pretreatment
With or Without Protamine and Subsequent Protamine Reversal of Anticoagulation

	Group I LMWH, Saline Pretreatment	Group II LMWH, Protamine Pretreatment	Group I LMWH, Saline Pretreatment	Group II LMWH, Protamine Pretreatment
	Antithrombin III (%)		α_2-Macroglobulin (mg/ml)	
Baseline values	85±5	86±8	-	-
After pretreatment	86±6	95±6	-	-
After LMWH	86±4	97±9	-	-
Before reversal (rev.)	83±5	88±5	4.4±0.5	3.7±0.3
1 minute after rev.	86±4	91±6	4.0±0.5	3.8±0.4
3 minutes after rev.	73±8	88±7	4.1±0.5	3.8±0.4
10 minutes after rev.	74±9	88±9	4.1±0.4	4.0±0.4

Data expressed as x ± SEM; n=6 in each group

TABLE 3.
AntiXa (Heparin IU/ml) Levels During Anticoagulation with Low Molecular Weight Heparin with or Without Pretreatment with Protamine Before Subsequent Protamine Reversal of Anticoagulation

	Group I LMWH, Saline Pretreatment	Group II LMWH, Protamine Pretreatment	Group III SH, Saline Pretreatment
Baseline	0	0	0
After pretreatment	0.02±0.03*	0.01±0.02	-
After heparin	1.09±0.13**	1.12±0.08**	1.46
Before reversal (rev.)	1.16±0.09**	1.23±0.14*	1.34
1 minute after rev.	0.81±0.11*	0.90±0.19*	0.06
3 minutes after rev.	0.72±0.14*	0.86±0.13*	0.08
10 minutes after rev.	0.98±0.14*	0.89±0.13*	0.01

Data expressed as x ± SEM; n=6 in Groups I and II, n=2 in Group III.
*p<0.01, **p<0.001
Comparison with Group III not possible because of small numbers.

TABLE 4.
C-1-esterase Inhibitor and C3 Levels During Anticoagulation With Low Molecular Weight Heparin or Standard Heparin and Pretreatment With or Without Protamine and Subsequent Protamine Reversal

	Group I LMWH, Saline Pretreatment	Group II LMWH, Protamine Pretreatment	Group III SH, Saline Pretreatment
	C-1-Esterase Inhibitor (%)		
Baseline	89±8	101±5	102
After pretreatment	91±9	130±13	-
After anticoagulation	121±23	165±28*	91
Before reversal (rev.)	89±16	136±23	71
1 minute after rev.	135±27	152±19	148
3 minutes after rev.	121±22	166±28	120
10 minutes after rev.	79±20	126±18	96
	C3 (%)		
Baseline	104±11	108±11	129
After pretreatment	99±12	106±10	-
After anticoagulation	107±13	105±10	130
Before reversal (rev).	101±8	102±7	133
1 minute after rev.	93±11	101±5	114
3 minutes after rev.	104±9	93±12	98
10 minutes after rev.	104±12	97±8	88

Data expressed as x ± SEM; n=6 in Groups I and II, n=2 in Group III; *p<0.05

TABLE 5.
6-keto-$PGF_{1\alpha}$ and TxB_2 Levels During Anticoagulation With Low Molecular Heparin and Pretreatment With or Without Protamine and Subsequent Protamine Reversal of Anticoagulation

	Group I LMWH, Saline, Pretreatment	Group II LMWH, Protamine Pretreatment	Group I LMWH, Saline Pretreatment	Group II LMWH, Protamine Pretreatment
	6-keto-$PGF_{1\alpha}$ (pg/ml)		TxB_2 (pg/ml)	
Baseline value	210±50	186±32	1173±245	739±133
After pretreatment	197±37	154±50	1185±287	1187±320
After heparin before reversal (rev.)	469±53*[a]	182±35	1284±226	815±193
1 minute after rev.	310±69	246±44	4813±1127*[a]	1782±515*
3 minutes after rev.	617±95**[a]	329±51	3412±1291	1493±489
10 minutes after rev.	475±81*[a]	283±35	1706±252	1136±268

Data expressed as x ± SEM, n=6 in each group; *p<0.05, **p<0.01,
[a]intergroup difference p<0.05

TABLE 6.
LTB_4 and LTC_4 Levels During Anticoagulation with Low Molecular Heparin and Pretreatment With or Without Protamine and Subsequent Protamine Reversal

	Group I LMWH, Saline Pretreatment	Group II LMWH, Protamine Pretreatment	Group I LMWH, Saline Pretreatment	Group II LMWH, Protamine Pretreatment
	LTB_4 (pg/ml)			LTC_4(pg/ml)
Baseline value	1025±192	1119±244	3365±231	3547±484
After pretreatment	952±178	883±186	3492±309	3358±255
After heparin before reversal (rev.)	1111±200	992±195	3630±385	3078±275
1 minute after rev.	1058±204	943±159	3998±656	3077±238
3 minutes after rev.	969±99	883±258	3342±145	2950±312
10 minutes after rev.	1057±254	1004±178	3213±191	3153±290

Data expressed as x ± SEM; n=6 in each group

TABLE 7.
Effects on Thrombin Clotting Time (TCT), Activated Partial Thromboplastin Time (APTT) and Bleeding Time During Anticoagulation With Standard Heparin or Low Molecular Weight Heparin and Pretreatment With or Without Protamine and Subsequent Protamine Reversal of Anticoagulation (Values Presented in Seconds)

	Baseline Value	2 Min After Anticoagulation	Before Reversal	After Reversal
	Group I (LMWH, Saline Pretreatment)			
TCT	6.2±0.2	53.6±8.5***	21.9±3.3**	6.8±0.3
APTT	13.6±1.5	36.1±13.0**	21.3±2.7*	13.6±1.1
Bleeding Time	130	385**		105
	Group II (LMWH, Protamine Pretreatment)			
TCT	6.8±0.4	10.4±1.0*	8.1±0.4*	6.0±0.3
APTT	13.6±0.8	17.6±1.5**	15.6±1.6*	14.3±1.3
Bleeding Time	120	400**		130
	Group III (SH, Saline Pretreatment)			
TCT	6.5±0.3	> 100***	> 100***	6.4±0.4
APTT	13.2±1.4	45.1±9.8***	35.8±8.8**	13.0±2.0
Bleeding Time	135	540**		115

Data expressed as x ± SEM; n=6 in each group
*p<0.05, **p<0.01, ***p<0.001; no intergroup differences

DISCUSSION

Administration of protamine to reverse heparin anticoagulation has been associated with a number of potentially serious hemodynamic and hematologic side effects (9). The action of protamine related to these events is only partially understood. Anaphylactic and allergic type reactions to protamine rarely occur (10), and other events have been implicated in most adverse reactions to protamine.

Platelets may be involved in the pulmonary and pulse effects. We have recently shown that systemic hypotension develops to the same extent in thrombocytopenic dogs as in those with normal platelet counts. However, the pulmonary artery hypertension and bradycardia usually accompanying these events was not seen (4). This suggests that the hypotension and pulmonary effects are mediated by different mechanisms. In fact, vasodilation appears responsible for much of the initial hypotension. Earlier work from our laboratory also has demonstrated that small amounts of protamine given prior to heparin anticoagulation and its subsequent reversal abolished adverse hemodynamic effects and diminished the thrombocytopenia, although the leukopenia was not reduced. The latter finding all but excludes the possibility that leukocytes themselves cause the adverse side effects attending protamine reversal of heparin (1).

Failure to document alterations in antithrombin III or α_2 macroglobulin levels in the current study suggests that thrombin generation or release of proteolytic enzymes is unlikely to be the major factor in causing the adverse effects of protamine. Other vasoactive substances, released for example from mast cells, could explain the effects of protamine administration. In this regard histamine release has been documented in these reactions (12), although histamine depletion or receptor blockage has not attenuated these responses (13). Results of C1-esterase assays in the present study preclude a major alteration within the bradykinin-system in these reactions, although some increase in C-1-esterase inhibitor levels was noted. Many investigators have suggested that complement activation by protamine mediates the adverse responses (14). With clinically relevant dosages of protamine in this experiment, as well as in earlier studies in dogs and humans, we have been unable to verify significant complement activation as the cause of the adverse effects (1,15).

This investigation revealed an increase in thromboxane B_2 after protamine reversal, perhaps a reflection of platelet aggregation and thromboxane release. 6-keto $PGF_{1\alpha}$ levels also increased after protamine administration. In this regard, cyclooxygenase inhibitors prevent pulmonary artery hypertension, but do not affect the other responses accompanying protamine reversal of heparin (15). Thus, it is unlikely that prostanoids are the major cause of protamine's side effects. The leucotrienes did not show any change, but the likelihood that such are uninvolved cannot be claimed inasmuch as leucotrienes are bound to cells and albumin, both of which were extracted in the assay performed in this experiment.

Another potential mechanism accounting for the action of protamine is that this basic protein alters cell membranes (10,17). SH has two specific binding sites on the endothelial cell membrane, with a higher affinity than LMWH (18). However, the adverse hemodynamic effects are not explained by this difference (2). Protamine reversal did not normalize the Anti-Xa levels achieved with LMWH. Protamine reversal of heparin normally causes a dissociation of heparin-antithrombin III complexes (3,19,20). If the short molecular chain of LMWH is unable to be dissociated, then the inability of Xa inhibition to be reversed can easily be explained.

In conclusion, this study has documented that protamine reversal of LMWH or SH anticoagulation does not significantly alter the complement, bradykinin or leucotriene systems, but does cause mild platelet activation, prostacyclin release, and has variable effects on Xa inhibition depending on the type of heparin initially administered. Protamine reversal of LMWH when compared to SH did not reveal any clear differences in eicosanoids or complement factors, although XaI activity clearly separated these two heparins.

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