Heparin-induced coagulopathy

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Intravenous heparin, at doses of 3.0 U./gm. of body weight, produced an intravascular coagulopathy in rats which was manifested by intestinal tract hemorrhage, a reduction in plasma fibrinogen concentration, a rise in fibrinogen-fibrin degradation products, and the absence of a rise in platelet count noted in the control animals. This coagulopathy could not be produced by conventional anticoagulant doses of heparin or the injection of large doses of heparin in the presence of protamine sulfate. Specific studies excluded hypoxemia, metabolic acidosis, and endotoxemia as possible etiologic factors. The coagulation abnormalities observed in this study differ from those produced by injection of other polyanionic substances but their precise pathogenesis is still uncertain.

Thrombocytopenia has been described during the administration of heparin in experimental animals[1–2] but similar blood changes were rarely observed in man.[3] While some reports attributed the apparent thrombocytopenia to counting artifacts,[4–7] other studies have documented a fall in blood platelets during heparin therapy and attributed this to idiosyncratic reactions, perhaps with an immunologic basis.[7–11] Recently, disseminated intravascular coagulation (DIC) has been reported in patients treated with heparin,[12] and in these cases the thrombocytopenia was accompanied by abrupt onset of hemorrhage, prolongation of the whole blood clotting time, decreased fibrinogen concentration, and a rise in fibrinogen-fibrin degradation products (FDP-fdp). This finding prompted us to perform a clinical study[13] which showed that thrombocytopenia occurred in 31 percent and DIC developed in 12 percent of patients receiving standard-dose heparin therapy. The present report demonstrates that similar changes can be produced by treating rats with large doses of heparin.

Materials and methods

Adult, male Wistar rats, weighing 200 to 250 grams (Microbiological Associates, Walkersville, Md.) were quarantined in air-conditioned quarters with laboratory chow and water available ad libitum for 2 weeks after delivery to insure absence of illness before studies were instituted. Heparin prepared from beef lung and porcine intestinal mucosa in concentrations of 1,000 U.S.P. units per milliliter, was supplied by the Upjohn Company, Kalamazoo, Mich. Arvin, a fractionation product of Malayan pit-viper (*Agkistrodon rhodostoma*) venom was obtained in a concentration of 100 units per

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milliliter from Twyford Laboratories, London, England. Urokinase derived from human urine was obtained from Sterling-Winthrop Company, Rensselaer, New York. Protamine sulfate solution containing 10 mg./ml. was supplied by Eli Lilly Company, Indianapolis, Indiana.

Hematocrit values were determined on blood collected in microcapillary tubes coated with ammonium and potassium oxalate. Platelet counts were performed on blood collected in glass tubes treated with ethylenediamine tetra-acetic acid (EDTA) and heparin (Drummond Scientific Company, Broomall, Pa.) according to the method of Bull, Schneiderman, and Brecker.[14] Clotting times were measured with the Lee and White[15] technique. The measurement of fibrinogen and fibrinogen-fibrin degradation products was made on plasma from blood collected in siliconized glass tubes (G. E. Silicone S.C. 87 Dic Film, General Electric Company, Waterford, N. Y.), with the use of 0.129M trisodium citrate (1 part citrate:9 parts whole blood) as the anticoagulant. Plasma was prepared by centrifuging whole blood at 1,100 × g for 15 minutes at 4° C. In control rats, fibrinogen levels were determined according to the method of Ratnoff and Menzie[16] and Allison's[17] technique, where Arvin is used instead of thrombin to clot fibrinogen. Only the latter technique was employed in quantifying fibrinogen concentration in heparin-treated rats.

To be certain that the reduction in plasma fibrinogen concentration and the elevation of FDP-fdp seen in rats treated with high doses of heparin were not artifacts, measurements were made on undiluted plasma samples after the in vitro addition of 650 U.S.P. U. of heparin/ml. (to provide a concentration greater than that achieved in the experimental animals). The standard thrombin–episilon–aminocaproic acid–calcium chloride mixture[18] and Arvin were compared in their capacity to remove fibrinogen from heparinized plasma. Similarly, FDP-fdp levels were determined in control and heparinized plasma before and after the addition of preformed FDP-fdp.

Fibrinogen-fibrin degradation products were measured by the tanned red cell hemagglutination inhibition immunoassay (TRCHII) as described by Merskey, Johnson, and Lalezari[18] modified for rat FDP-fdp with the use of formalinized and tanned human type O–negative erythrocytes coated with purified rat fibrinogen.[19] Results were expressed as the highest serum dilution which prevented agglutination of fibrinogen coated red cells by anti–rat fibrinogen antibodies. Anti–rat fibrinogen antiserum was prepared by injecting 0.01 mg. of purified rat fibrinogen emulsified in complete Freund's adjuvant into each of the footpads and two subcutaneous sites in New Zealand white rabbits at weekly intervals. Immune serum was collected 2 weeks after the last injection and absorbed three times with aged, normal rat serum at 25° C. over 36 hours. The absorbed antiserum produced a single precipitin band when tested against rat fibrinogen in gel diffusion and a 1:10,000 dilution completely agglutinated erythrocytes coated with rat fibrinogen.

Arterial blood determinations for pH, Pco_2, and Po_2 were obtained in a separate panel of rats at 1, 2, 4, and 6 hours after the heparin injections. Arterial blood was collected under oil from the abdominal aorta and studied with a PHM 71 MK-2 microsystem acid-base analyzer (Radiometer Corporation, Copenhagen, Denmark). Blood pressure in all groups was monitored by the indirect tail-cuff method with a strain gauge plethysmograph (Narco Biosystems, Houston, Texas).

Each heparin preparation used in this study was tested for endotoxin by means of the Limulus lysate assay of Levin and Bang.[20] Aliquots of blood obtained from rats before and 2 hours after injecting 3.0 U. of heparin/gm. were tested for endotoxin and cultured in nutrient broth and on blood agar plates for 48 hours at 37° C.

Slices from each organ were snap-frozen in methyl butane and Dry Ice and sectioned at 8×10^{-6} M. in a cryostat. After being dried in a vacuum with calcium sulfate desiccant for 30 minutes at room temperature, the sections were defatted in acetone and stained with fluorescent anti–rat fibrinogen (Cappel Laboratories, Downingtown, Pa.) for 1 hour at 25° C. After being washed and mounted in a 0.1M NaH_2PO_4–0.1M NaH_2PO_4–0.85 gm./100 ml. of sodium chloride-glycerol solution (1:9, v/v), the sections were examined with a Leitz fluorescent microscope. The activity and specificity of this antiserum were confirmed by double-gel diffusion and specific staining of a three times washed (0.85 gm./100 ml. of sodium chloride) clot prepared from recalcified rat plasma. Additional tissue samples from the same organs were fixed in buffered formalin, embedded in paraffin, and sectioned at 5×10^{-6} meter. Alternate sections were stained with hematoxylin and eosin, Fraser-Lendrum's fibrin stain, or periodic acid–Schiff reagent[21] in an attempt to demonstrate intravascular fibrin deposits. Mean, standard deviation, standard error of the mean, and comparative group analysis were computed by conventional methods.[22]

Methods of procedure. In these experiments the rats were restrained in Bollman-type cages[23] to facilitate collection of blood samples at sequential intervals without the use of anesthetics. Heparin

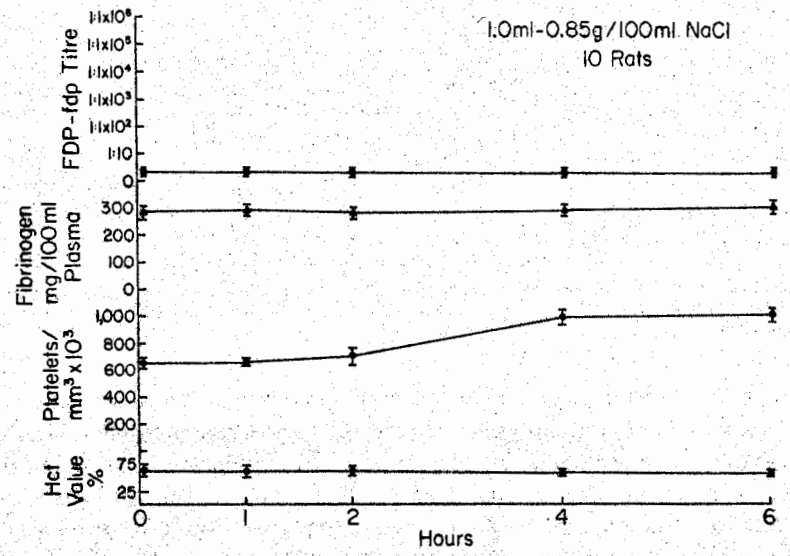

Fig. 1. Ten rats (controls) were injected intravenously at time zero with 1.0 ml. of solution of sodium chloride. Note the rise in platelet count observed beginning at 2 hours. Each point is the mean value and the barred line is the S.E.M.

was injected intravenously into separate groups of rats at doses of 0.3, 1.0, 2.0, and 3.0 U.S.P. U./gm. of body weight. Each dose was diluted in sterile 0.85 gm./100 ml. of sodium chloride to a final volume of 1 ml. which was injected over 1 to 2 minute intervals. Control rats were given 1 ml. of 0.85 gm./100 ml. of sterile sodium chloride intravenously. Blood samples were obtained immediately before heparin injection and serially at 1, 2, 4, and 6 hours thereafter by transecting a tail vein and allowing the blood to flow directly into the containers described. These sequential studies were performed on a total of 2.5 to 3.0 ml. of blood to minimize volume depletion artifacts and replicate panels of animals were used when necessary. All rats were necropsied at the termination of the experiments. Histologic evidence of DIC was sought by killing two rats from each experimental group at the sequential time intervals designated above and examining their lungs, liver, spleen, kidneys, and lymph nodes by light and fluorescence microscopy.

Results

Commercial preparations of beef lung and porcine mucosal heparin were evaluated separately in these studies. Since no differences were observed in their in vivo effects, data from both experimental groups were combined for presentation in subsequent sections of this report.

Control animals. Intravenous injection of 1.0 ml. of sodium chloride solution into 10 rats did not alter the mean systolic blood pressure, arterial blood pH, Pco_2, Po_2, clotting time, hematocrit value, plasma fibrinogen concentration, or FDP-fdp. Beginning at 2 hours, the mean platelet count began to rise from a control value of 650,000/mm.^{3} to a peak mean value at 4 hours of 1,000,000/mm.^{3}, which persisted for the duration of the study (Fig. 1). At the completion of the study, all animals appeared healthy and no significant abnormalities were found at necropsy.

Effect of 0.3 to 2.0 U. of heparin/gm. of body weight. The intravenous administration of 1.0 ml. of solution containing heparin 0.3 U./gm. of body weight to separate groups of five rats per group did not alter the mean systolic blood pressure, the arterial blood pH, Pco_2, Po_2, hematocrit value, and plasma fibrinogen concentration. In the ani-

Table I. Mean systolic blood pressure (mm. Hg)*

Dose of heparin (U./gm.)	Time after heparin injection						
	0	10 min.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
3.0	104	77	69	26	28	22	NR†
2.0	94	95	73	70	64	53	48
0.3	94	99	100	102	101	102	104

*The mean systolic blood pressure is shown in three groups of animals (five rats per group), each receiving a different dose of heparin.
†NR = not recordable.

mals receiving heparin 0.3 or 1.0 U./gm., FDP-fdp did not increase. In the animals receiving 2.0 U/gm. there was a gradual fall in mean systolic blood pressure to 48 mm. Hg at 6 hours (Table I). In this group of animals, small quantities of FDP-fdp were detected in the serum at 2, 4 and 6 hours but the arterial blood pH, Pco_2, Po_2, hematocrit value, and plasma fibrinogen concentration were unaltered. A rise in platelet count, beginning at 2 hours and of the same magnitude as seen in the control animals, was noted in all groups of animals receiving 0.3, 1.0, and 2.0 U. of heparin/gm. of body weight. The clotting time was prolonged to 60 to 90 minutes in the group of animals receiving 0.3 U. of heparin/gm. and to more than 12 hours in all animals receiving 1.0 and 2.0 U./gm. of body weight. At the end of the study, all animals appeared healthy. At necropsy the findings were unremarkable except in those receiving 2.0 U. of heparin/gm., where focal gastrointestinal hemorrhage was found.

Effect of 3.0 U. of heparin/gm. of body weight. The intravenous administration of 1.0 ml. of heparin containing 3.0 U./gm. of body weight caused a marked prolongation of the clotting time to greater than 24 hours. The mean systolic blood pressure fell within 10 minutes after injection and continued to decline to nonrecordable levels over the course of the experiment (Table I). The hematocrit value decreased moderately but progressively in these animals. The platelet count rise seen in all other treatment groups and the control group was not seen in these rats (Fig. 2). The plasma fibrinogen concentration fell to 40 to 50 percent of the preheparin levels. Fibrinogen-fibrin degradation products increased to very high concentrations with peak values at 2 hours and remained elevated throughout the duration of the study (Fig. 2). The difference in the alterations in platelet count, plasma fibrinogen concentration, and FDP-fdp titer seen in this group of animals compared to all other groups was significant at the 0.0001 level. In this group of 15 animals the arterial blood pH, Pco_2, and Po_2 were normal throughout the duration of the experiment (Table II). All animals in this treatment group died 6 to 18 hours following the injection of heparin. At necropsy, fresh hemorrhage (not massive) was present in the alimentary tract in 13 of 15 animals, but there were no signs of potentially lethal hemorrhage at any other site. Intraperitoneal and retroperitoneal accumulations of blood were not present. Necropsy studies in combination with blood pressure measurements of rats killed at sequential intervals indicated that the hypotension in this group commenced 2 to 3 hours before the first sign of hemorrhage in the alimentary tract.

Effect of 3.0 U. of heparin and 0.3 mg. of protamine sulfate/gm. of body weight. The intravenous administration of 1.0 ml. volume containing 3 U. of heparin/gm. of body weight immediately followed by 0.5 ml. of a solution containing 0.3 mg. of protamine sulfate/gm. of body weight in five rats did not alter the mean systolic blood pressure or the arterial blood pH, Pco_2, or Po_2. The clotting time at 1 hour was normal and un-

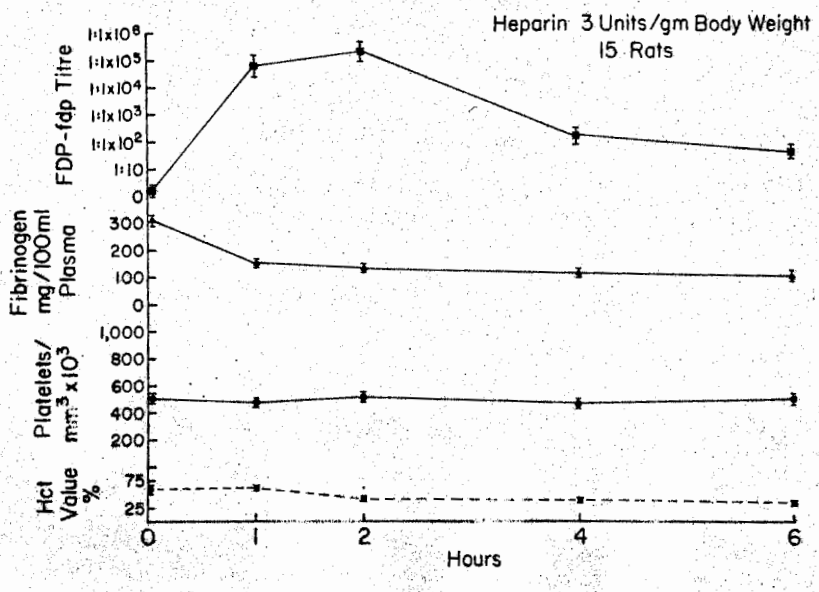

Fig. 2. A group of 15 rats were injected intravenously with 3.0 U. of heparin per gram of body weight. The rise in platelet count observed in the controls and in the animals treated with heparin followed by protamine is not observed in these animals. Each point is the mean value and the barred line is the S.E.M.

Table II. Mean arterial blood changes with heparin, 3.0 U. per gram of body weight*

	Time after heparin injection					
	0	*10 min.*	*1 hr.*	*2 hr.*	*4 hr.*	*6 hr.*
pH	7.42	7.43	7.41	7.38	7.49	7.46
Pco_2	35	36	41	44	31	25
Po_2	75	91	90	87	90	83

*The mean arterial blood gas and pH changes before and following the intravenous injection of heparin are shown for a group of 15 rats.

changed from the preheparin clotting time. The hematocrit value and plasma fibrinogen concentration were unchanged from the preheparin studies. The rise in platelet count seen in the control animals and in those animals which received 0.3, 1.0, and 2.0 U. of heparin/gm. was also noted in these animals (Fig. 3). No FDP-fdp appeared in the blood. At the completion of the study period all animals were healthy and at necropsy no gross abnormalities were observed.

Effect of 0.3 mg. of protamine sulfate/gm. of body weight. The intravenous administration of 1.0 ml. of protamine sulfate containing 0.3 mg./gm. of body weight of five rats gave results identical to those described in the control group. At necropsy no abnormalities were noted.

Histologic studies

Histologic examination with routine and special stains revealed neither fibrin nor platelet thrombi in the vessels of any of the heparin-treated or control animals. Immunofluorescent studies did not demonstrate fibrin deposits in these rats. Histologic abnormalities were seen only in the group of animals receiving 3.0 U. of heparin/gm. of body

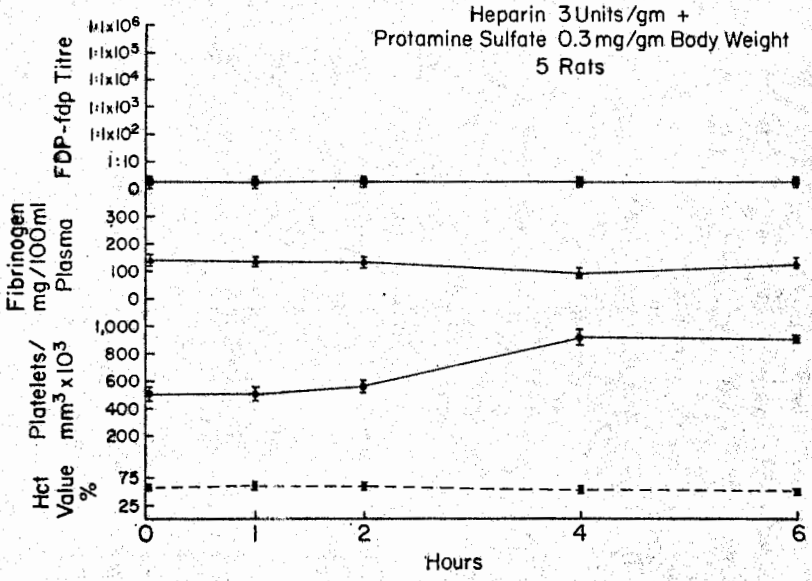

Fig. 3. A group of five rats were injected intravenously with 3.0 U. of heparin per gram of body weight immediately followed by 0.3 mg. of protamine sulfate per gram. The alterations noted in the rats which received 3.0 U. of heparin per gram alone are not observed in these animals. The rise in platelet count seen in the controls is also observed in this group. Each point is the mean value and the barred line is the S.E.M.

weight. In this group, vascular congestion and focal extravasation of blood were observed in the kidneys and lymph nodes in 13 of 15 animals.

In vitro determination of fibrinogen and FDP-fdp in the presence of heparin. The antithrombin properties of heparin prevented the use of conventional methods of measuring fibrinogen as thrombin-clottable protein in this study. As indicated by the data in Table III, Arvin can be used to determine accurately the fibrinogen concentration in plasma specimens containing heparin. Despite the presence of high concentrations of heparin, Arvin can remove completely all clottable proteins without generating FDP-fdp or interfering with the immunologic determination of FDP-fdp. As shown in Table III, when Arvin, as contrasted with thrombin, was employed, the concentration of fibrinogen and the titer of FDP-fdp were not altered by heparin.

Endotoxin studies

Endotoxin could not be detected in the heparin aseptically removed from the administration vials nor in the blood of the rats studied before and 2 hours after the intravenous administration of 3.0 U. of heparin/gm. No bacterial growth was observed from the blood of animals in the nutrient broth or on blood agar plates.

Discussion

The mechanism of heparin-induced thrombocytopenia has never been adequately explained. Reports attributing this phenomenon to direct toxic effects upon platelets or heparin-antibody-platelet interactions have not been substantiated.[11, 25] Although it has been suggested that some subjects developing thrombocytopenia during heparin administration may be experiencing an intravascular coagulopathy,[12] most reports have not in-

Table III. Comparison of fibrinogen concentrations and FDP-fdp titers*

	I Rat plasma		*II Rat plasma, 650 U. of heparin*		*III Rat FDP-fdp (1:2048) + 10 U. of Arvin*	*IV Rat plasma + FDP-fdp (1:2048), 650 U. of heparin + 10 U. of Arvin*
	Thrombin	*Arvin*	*10 U. of arvin*	*Thrombin*		
Fibrinogen (mg./100 ml.)	155	157	153	No clot	0	82
FDP-fdp titer	0	0	0	1:524,288	1:2048	1:1024

*Comparison of fibrinogen concentration and FDP-fdp titers in aliquots of control and heparinized rat plasma with thrombin and Arvin (Column I). Heparin prevented clot formation in the thrombin assay, causing a false elevation in FDP-fdp when the nonclotted fibrinogen reacted with antibody (Column II). In contrast, Arvin removed all clottable proteins from both normal and heparinized plasma without altering the titers produced by adding known quantities of preformed FDP-fdp to the assay (Columns III and IV). This indicates that both fibrinogen and FDP-fdp levels can be accurately determined in heparinized plasma with the use of Arvin.

cluded studies required for documenting this process. Recognition of a DIC-like disorder in the anticoagulated patient is dependent upon demonstrating a fall in blood platelets and fibrinogen levels accompanied by a rise in FDP-fdp. In a recent study of 52 patients receiving standard doses of heparin[13] we observed thrombocytopenia (<100,000 platelets/mm.^{3}) in 16 subjects. Ten of these patients showed elevated levels of FDP-fdp during heparin therapy (no FDP-fdp were detectable prior to treatment) and in five subjects the plasma fibrinogen concentration fell to 25 to 50 percent of the pretreatment levels. These data suggest that such an intravascular coagulopathy may contribute to the significant incidence of bleeding seen with this anticoagulant.

The data in the present study demonstrate that the onset of bleeding, fall in blood pressure, reduction in plasma fibrinogen concentration and platelet count, and rise in FDP-fdp occurred in rats during the administration of heparin but only with amounts far exceeding those used in clinical medicine. The abnormalities could not be produced in the presence of protamine sulfate, a heparin antagonist. Intravenous injection of large doses of heparin into rats caused prolonged clotting times, gastrointestinal hemorrhage, and hypotension which could be attributed to the anticoagulant effects of heparin. However, the demonstration of a relative thrombocytopenia (failure to exhibit the rise in platelet counts seen in other treatment groups and controls), fall in plasma fibrinogen levels, and concomitant rise in FDP-fdp after injecting 3 U. of heparin/gm. of body weight suggested that these animals were experiencing a DIC-like disorder. This thesis was supported by documenting that the reduction in plasma fibrinogen levels and rise in FDP-fdp were not artifacts caused by the antithrombin effects of heparin during in vitro assay.[17, 21]

We were unable to detect intravascular fibrin deposits or platelet thrombi in tissues from these animals, but there is evidence that such deposits can be completely absent in some well-documented cases of DIC.[26]

Several different mechanisms were considered as possible causes of the intravascular coagulopathy in these animals. Since all of the rats appeared clinically well at the start of these experiments and no evidence of significant disease was found at necropsy, it is unlikely that this coagulopathy was related to latent infection. The occurrence of the intravascular coagulopathy only in rats given 3 U. of heparin/gm. of body weight suggested that this might be a dose-related, direct toxic effect. In vitro studies have shown that intestinal mucosal heparin may have adverse effects on platelets which are not pro-

duced by beef lung heparin.[27] In the present study both types of heparin were found to be equally potent in producing the intravascular coagulopathy. This indicated that the platelet-aggregating properties produced by intestinal mucosal heparin in vitro could not be correlated with the reduction in platelets seen in vivo. Since heparin is prepared from tissues rich in thromboplastic activity, it seemed plausible that thromboplastic contaminants or preservatives in the commercial heparin preparations might cause this disorder. A specific protease that could activate the coagulation system or destroy platelets and fibrinogen may be in the preparation. Such a contaminant may not be significant in routine doses of heparin but would be magnified at the doses employed in this study. However, sequential injections of heparin and protamine sulfate failed to induce coagulation abnormalities in test animals. Endotoxin has been implicated as a cause of DIC[28] but we were unable to detect endotoxin in either the heparin or in the blood of the animals before or after treatment with heparin. As heparin is strongly acidic, it was postulated that the concentrations employed in these studies (10 to 15 times the usual therapeutic dose) might cause a metabolic acidosis with secondary alterations in blood pressure and activation of the coagulation system. Hypoxemia was also considered as a possible mechanism since DIC has been reported in this setting.[23, 29] These possibilities were excluded when consistently normal arterial pH and blood gases were found in heparin-treated rats. Hypotension is a possible cause and cannot be excluded. However, alterations in FDP-fdp and fibrinogen were noted at 1 hour when no striking fall in blood pressure had occurred. Since previous investigators have described anticomplementary effects,[30–36] impaired phagocytosis,[37] and decreased lymph node–filtering capacity[38] in heparinized rats, it was postulated that these changes could permit hematogenous dissemination of bacteria or bacterial products from the intestine and result in such a coagulopathy. Blood cultures were sterile and studies for endotoxin were negative in the present study, suggesting that this mechanism is unlikely.

While heparin is a highly charged polyanion capable of reacting with proteins and cell surfaces in vitro and in vivo,[39, 40] there is no convincing evidence that such interactions directly activate the coagulation system. Intravenous infusions with other polyanions (polyanethol sulfonate) have been reported to activate the clotting system and cause renal cortical necrosis in rats.[41] However, this appears to be a secondary event related to platelet aggregation and endothelial damage.[42] Since this form of intravascular clotting can be minimized by heparin therapy,[43] it appears to differ from the intravascular coagulopathy described in the present study. Other investigators have suggested that polyanions may activate the complement cascade which triggers intravascular coagulation.[43, 44] This concept is based upon observations that preincubation of serum with heparin results in loss of complement components C_1, C_2, and C_3-C_9 activity,[45] but it is still uncertain whether these changes reflect activation of the complement sequence or anticomplementary effects due to heparin-protein binding. The relevance of these in vitro findings to intravascular coagulation must be questioned. Rent and associates[45] have shown that simultaneous addition of heparin and protamine enhances the loss of complement activity in serum samples. While we have no data on complement consumption in the present study, our results clearly indicate that sequential injections of heparin and protamine in vivo failed to induce clotting abnormalities.

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