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ENDOGENOUS ANTICOAGULATION DURING EXTRACORPOREAL PERFUSION IN T--ETC(U)

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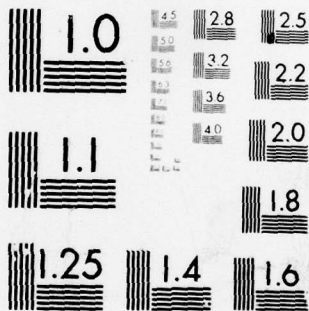
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TECHNICAL REPORT NO. 139

ENDOGENOUS ANTICOAGULATION DURING EXTRACORPOREAL PERFUSION IN THE DOG:
GENERATION OF A HEPARIN-LIKE INHIBITOR

T. L. Murphy, F. J. Walker, F. B. Taylor III, B. Beller-Todd,
L. T. Archer, S. S. Sofer, and L. B. Hinshaw

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ABSTRACT

Studies were done to define the coagulation defect that develops in anesthetized dogs perfused with an arteriovenous extracorporeal perfusion system without added heparin. The development of whole blood clotting times (WBCT) greater than 24 hours is associated with the appearance of a plasma inhibitor of thrombin and Factor Xa clotting of bovine plasma. This inhibitor stimulated the inactivation of Factor Xa by antithrombin III (ATIII) but not by o-methyl-isourea modified ATIII. Thrombin inhibition by ATIII was also stimulated. Six perfused dogs developed an equivalent of 0.98 to 6.15 U/ml of heparin activity determined by inhibition of thrombin-induced clotting of normal canine plasma. This activity was heat stable, adsorbed by BaSO₄ and neutralized by protamine. Infusion of protamine sulfate into two perfused dogs reversed the anticoagulant activity and brought the WBCT from greater than 24 hours to less than control. Five eviscerated dogs in which the hepatic artery was ligated developed peak plasma heparin activity of 3.2±1.0 U/ml. We conclude that dogs perfused on our extracorporeal perfusion system without added heparin develop an endogenous heparin activity in their plasma which is a major contributor to their autoanticoagulated state. In addition, this heparin activity may have an extra-hepatic origin.

INDEX WORDS: endogenous heparin; antithrombin III, cardiopulmonary bypass; hemodialysis

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INTRODUCTION

The problem of preventing blood from clotting during the extracorporeal perfusion of either organs (3) or whole animals (1) has been solved by adding exogenous anticoagulants. Heparin was used during the development of hemodialysis (23) and cardiac bypass techniques (12) in animals. Heparin has continued to be used in humans since the introduction of these procedures into clinical use (13,20).

To determine if systemic heparinization was necessary for successful extracorporeal perfusion, recent studies have been performed with perfused, nonanticoagulated dogs (4,5,11). The first of these studies established that dogs could tolerate 24 hours of perfusion with a circuit containing a membrane oxygenator without added heparin (11). These results encouraged our laboratory to attempt extracorporeal perfusion in dogs without heparin. Our results demonstrated that this procedure was well tolerated with good surgical hemostasis and maintenance of blood pressure (4,5).

One unexplained finding of these studies was the failure of whole blood to clot after the dog had been perfused at pump flow rates exceeding 800 ml/minute for 45 minutes (4,5). This was not accompanied by severe depletion of platelets and fibrinogen (15), changes which might be expected to occur if the coagulation defect was due to disseminated intravascular coagulation (24) or pathological fibrinolysis (21). These results led to a search for other factors that might be involved. The characterization of an inhibitor which appears to be the major cause of the coagulation defect produced with our perfusion system is the subject of this paper.

MATERIALS AND METHODS

The extracorporeal perfusion system: general description of procedure

The details of the extracorporeal perfusion system are fully described in a companion paper (15). Adult mongrel dogs weighing from 15 to 25 kg were anesthetized with 25 mg/kg of sodium pentobarbital, intubated with an orotracheal tube, and allowed to breathe spontaneously. The arteriovenous perfusion circuit which carried blood from the femoral arteries to femoral veins consisted of flexible Tygon plastic tubing, a Nalgene plastic reservoir, and a calibrated roller-type pump.

A constant level of blood was maintained in the reservoir by adjusting screw clamps on the arterial outflow tubing leading to the reservoir while pump flow rate was constant. The animal was perfused under anesthesia for 3 hours and observed for 3 hours following termination of the perfusion. No animal received an anticoagulant of any kind. All catheters and pressure transducers were maintained with normal saline.

Evisceration with ligation of the hepatic artery

To investigate the role of the abdominal viscera, especially the liver, in the production of the coagulation defect, anesthetized dogs were eviscerated and the hepatic artery ligated by a previously described technique (2,14). In short, the stomach, intestines, pancreas and spleen were removed. The hepatic artery was then ligated. Within 20 minutes after evisceration, the extracorporeal perfusion was started.

Collection of blood and preparation of plasma

Samples of blood were drawn into a plastic syringe from a polyethylene cannula in the right brachial artery. Three ml were used to perform modified Lee-White clotting times. The remainder was anticoagulated with Na citrate (0.38% final concentration) or Na oxalate (0.01 M final concentration) in plastic culture tubes. Platelet-rich plasma was prepared from the citrated

whole blood by centrifugation at 1000 g for 60 seconds. Platelet counts were performed with a Coulter automatic particle counter and platelet morphology examined by phase contrast microscopy. Plasma was prepared by centrifugation at 3000 g for 15 minutes and stored at -20°C in plastic culture tubes until the remaining coagulation and heparin assays were performed.

Normal control plasma was obtained from fasting beagle dogs by jugular venipuncture using a two-syringe technique. The blood was anticoagulated with Na citrate (0.38% final concentration) or Na oxalate (0.01 M final concentration). Plasma was prepared and stored at -20°C .

To determine if the plasma heparin activity could be removed by barium sulfate adsorption, 50 mg of BaSO_4 per ml of oxalated plasma was added and stirred for 15 minutes at room temperature. The BaSO_4 was precipitated by centrifugation at 3000 G for 10 minutes. The adsorbed plasma was decanted and assayed for heparin activity.

The in vitro sensitivity of the heparin activity to neutralization by protamine sulfate was determined in the following manner. The stock solution of protamine was diluted in 0.9% NaCl to give a final concentration of 200 $\mu\text{g}/\text{ml}$. From 0.01 to 0.1 ml of this solution was added to 1.0 ml samples of citrated plasma drawn before and after 20 and 90 minutes of extracorporeal perfusion. The heparin activity in the sample was then measured using a modified thrombin clotting time of normal canine plasma. The minimum amount of protamine needed to correct the thrombin time back to preperfusion values was recorded.

Proteins

Factor X was purified from a barium eluate of barium adsorbed bovine plasma as described elsewhere (25). Fibrinogen was purified from barium

adsorbed plasma by the method of Straughn and Wagner (29). Antithrombin III was purified from barium adsorbed plasma as described (10). Thrombin was prepared by activating purified prothrombin with purified Factor Xa, Factor V, crude brain cephalin and calcium chloride as described previously (25). Activated Factor X was prepared by reacting Factor X with Factor X-activator from Russell's viper venom and calcium as described elsewhere (25). The lysine residues on antithrombin III were modified according to the method of Rosenberg and Damus (28). The modified protein retained 70% of its inhibitory activity against thrombin. The active modified species lost 50% of its sensitivity to heparin (31).

Coagulation assays

Modified Lee-White whole blood clotting time. Three 1.0 ml samples of whole blood drawn from the brachial artery of the experimental animal were placed in 12x75 mm glass tubes. The tubes remained at room temperature and the first tube was tilted at intervals of 30 seconds until a solid clot formed. The second and third tubes were then treated similarly in sequence. The clotting time was the time required for a solid clot to form in the third tube (22).

Fibrinogen level. The fibrinogen level was determined by a thrombin clotting time as described by Hougie (16). The clotting time was performed at 37°C by adding 0.1 ml of thrombin (bovine, 100 U/ml) to 0.2 ml test plasma diluted 1:10 with barbital-buffered saline (pH 7.35, 0.0285 M). A standard curve was made using a commercially prepared fibrinogen solution (Data-Fi, Dade Diagnostics) diluted with barbital-buffered saline (pH 7.35, 0.0285 M). Clotting times were performed in duplicate and the mean used to determine the fibrinogen concentration from the standard curve.

Heparin assays

Modified thrombin clotting time. A modification of the heparin assay based on prolongation of the thrombin clotting time (26) was used to measure heparin activity. A thrombin clotting time was performed in the following manner. Normal citrated plasma was warmed 3 minutes in a 37°C water bath in 10x75 mm glass tubes. Then 0.05 ml test plasma was added followed by 0.01 ml thrombin diluted in 0.1 M CaCl₂. A standard curve was prepared using sodium heparin (beef lung, 1000 U/ml) in normal citrated plasma.

The inhibition of Factor Xa-initiated clotting of plasma was measured in a one-stage assay. 0.1 ml phospholipid (500 µg/ml), 0.1 ml CaCl₂ (0.025 M), 0.05 ml Factor Xa (0.1 µg/ml) and 0.05 ml of the inhibitor sample were mixed at 37 °C. Clotting was initiated by the addition of 0.1 ml of oxalated bovine plasma. A standard curve was derived by measuring the clotting time at various Factor Xa concentrations. The amount of Factor Xa remaining at a given time was measured by the same method except that no inhibitor was added in the assay. All of the reagents in the assay were made in the 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, and 1 mg/ml bovine serum albumin.

Thrombin activity was also measured by a fibrinogen clotting assay. The thrombin sample was made to a final volume of 0.3 ml in buffer (0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, 1 mg/ml bovine serum albumin). Clotting was initiated by the addition of 0.1 ml of fibrinogen (10 mg/ml). A thrombin standard curve was constructed by measuring the clotting time at various thrombin concentrations.

RESULTS

The whole blood clotting times (WBCT) of dogs perfused without added heparin on an arteriovenous extracorporeal perfusion system became prolonged at blood flows of 800-1100 ml/minute. The lengthening of the WBCT occurred progressively, reaching the value of greater than 24 hours by 45 minutes of

perfusion. Following termination of the extracorporeal perfusion, the whole blood clotting time returned to normal in 3 hours (Figure 1).

A prolongation of the WBCT could be caused by an alteration of platelets, a deficiency of coagulation factors, or an inhibitor of coagulation. The effects of perfusion with our system on the prothrombin time, partial thromboplastin time, fibrinogen level, Factor V, VIII and X levels, fibrin degradation products, platelet count, and platelet aggregation are described in a companion paper (15). To determine if an inhibitor of coagulation was released, we examined the effect of plasma from a perfused dog on thrombin-initiated clotting of normal canine plasma and observed that plasma from the perfused dog inhibited thrombin clotting of normal canine plasma. Using commercial heparin as a standard for comparison, we observed the peak inhibitor activity to vary between 0.8 to 6.2 U/ml in six different dogs with a mean of 3.7 U/ml. Three hours after the termination of perfusion, the anticoagulant activity was undetectable in four of six dogs (Figure 2).

The inhibitor was not specific for dog plasma. Prior to extracorporeal perfusion, dog plasma had no effect upon the clotting of bovine plasma initiated either with bovine thrombin or bovine Factor Xa. After 20 minutes of perfusion, small amounts of the perfused dog plasma would inhibit the clotting of either thrombin or Factor Xa-initiated clotting of bovine plasma. The dilution curves of the inhibitor were similar in each assay (Figure 3). In addition, the dilution curve of a similar amount of heparin in a Factor Xa-initiated clotting assay was similar to that with perfused dog plasma.

Since it is possible in each of these assays that the inhibitor might interfere with the thrombin interaction with fibrinogen, we examined the effect of the inhibitor on individual clotting factors. In the absence of

plasma or any other factors, low levels of the dog plasma had no effect upon Factor Xa. However, in the presence of antithrombin III we observed a large stimulation of antithrombin III inhibition of Factor Xa. Stimulation of antithrombin III inhibition of serine proteases is a characteristic property of heparin (28). In order to substantiate that this inhibitor is acting through a heparin-like mechanism, we used o-methylisourea derivatized antithrombin III. This modification of antithrombin III alters the lysine residues, reducing the affinity between antithrombin III and heparin. The dog plasma did not stimulate the inhibition of Factor Xa by the modified antithrombin III (Figure 4). As observed with Factor Xa, small amounts of perfused dog plasma stimulated the inhibition of thrombin by antithrombin III (Figure 5), which is analogous to heparin.

Two other properties of the inhibitor were observed which indicate that it is similar to heparin. First, when protamine sulfate was incubated in plasma from perfused dogs, the ability of that plasma to inhibit thrombin-initiated clotting of normal dog plasma was blocked. Between 0.75 to 1.0 mg of protamine sulfate blocked the expression of 100 units of inhibitor activity. Second, we found that barium adsorption of oxalated plasma removed the inhibitor. Similar experiments with heparin indicated that it too could be removed by barium adsorption.

We have observed several properties of this factor that distinguish it from heparin. First, it did not react with Azure A, as measured by a shift in the visible spectrum of the dye. In contrast, an equivalent amount of heparin (on the basis of anticoagulant activity) added to control dog plasma did cause a shift in color. Second, we observed that the anticoagulant activity was precipitable by 5% (W/V) trichloroacetic acid. In contrast,

heparin remained soluble in control experiments. This material was insensitive to boiling. When dog plasma was boiled for two minutes, no loss of the anticoagulant activity was observed. Following boiling the precipitated protein was centrifuged and the supernatant assayed. The anticoagulant activity was observed to be pelleted with the protein. This contrasts with our observation that when heparin was added to plasma, boiled and centrifuged, the activity remained in the supernatant. These observations indicated that the anticoagulant factor may be tightly bound to protein.

To determine if our in vitro observations would apply in vivo, we infused protamine sulfate into two dogs that had been perfused on the extracorporeal system for 90 minutes. In a 19 kg dog, 13 mg of protamine sulfate infused into the jugular vein over 9 minutes reduced the inhibitor activity from 1.2 U/ml to zero and shortened the whole blood clotting time from greater than 24 hours to 3 minutes. In a 16 kg dog, 26 mg of protamine sulfate infused over 24 minutes decreased the inhibitor activity from 3.7 U/ml to zero and shortened the WBCT from greater than 24 hours to 2.5 minutes.

Since the inhibitor appeared to function in a manner similar to heparin, we were concerned about the source of this material. Since polymers used in plastics are reported to inhibit blood coagulation in a manner similar to heparin (31), we considered the possibility that the inhibitor was being leached from the plastics used in the perfusion system. However, when either normal saline, citrated whole blood, or nonanticoagulated whole blood was circulated in the perfusion circuit for 30 minutes, no inhibitor activity could be detected.

A second possible source of the inhibitor might be the liver since it is well established that dog livers contain large amounts of heparin (19). In order to determine the contribution of the liver and other abdominal viscera to the inhibitor activity, the dogs' intestines, stomach and spleen

were removed and the hepatic artery ligated prior to perfusion on the extracorporeal system. Five of six dogs treated in this fashion developed an inhibitor to thrombin-initiated clotting of normal plasma (Figure 6). The average peak level of inhibitor activity was 3.2 ± 1.0 U/ml, which was not significantly different from the levels observed in normal perfused dogs (3.7 ± 1.7 U/ml). One of the eviscerated dogs with the hepatic artery ligated developed no plasma heparin activity, had clots form in the reservoir, and died after 120 minutes of perfusion. The inhibitor activity which developed in the other five dogs was adsorbed by BaSO_4 and reversed in vitro by protamine sulfate, as measured by the effect of samples on the thrombin clotting time of normal canine plasma. Despite peak plasma heparin activity of 1.7 to 4.8 U/ml in this group of dogs, the whole blood clotting time was never greater than 24 hours as it was in the noneviscerated perfused dogs. By 45 minutes of perfusion, it was less than twice the normal value (Figure 7).

DISCUSSION

Studies in our laboratory have previously shown that dogs perfused with our extracorporeal system without added heparin develop an autoanticoagulated state (4,5). The current study demonstrates that these perfused dogs develop a circulating inhibitor of Factor Xa and thrombin-induced clotting of normal canine plasma. This activity was "heparin-like" since it required antithrombin III for expression. Since other glycosaminoglycans such as chondroitin polysulfates (8) and dermatan sulfate (30) can have anticoagulant properties, the exact structure of the "heparin-like" compound will require further study.

The anticoagulant activity formed in perfused dogs has several properties that differentiate it from fibrin/fibrinogen degradation products. This can be inferred from an experiment of Kowalski who, following infusion of the

plasminogen activator streptokinase into dogs, found an inhibitor of thrombin clotting of normal plasma (21). This inhibitor, presumably fibrin degradation products, was found to be heat-labile and could not be adsorbed from plasma by barium sulfate. This differs from both heparin and the inhibitor in the plasma of our perfused dogs which are both heat-stable and are adsorbed by barium sulfate.

The heparin activity appears to be an important factor in the development of autoanticoagulation produced in perfused dogs. Infusion of protamine sulfate caused plasma levels of the heparin activity to fall to undetectable levels. This decrease was associated with a shortening of the whole blood clotting time from greater than 24 hours to less than 4 minutes. This correction of the clotting defect by protamine, plus the lack of a significant decrease in the platelet count and fibrinogen level (15), suggests that decreased levels of clotting factors was not the major cause of the prolonged whole blood clotting. In dogs perfused with a membrane oxygenator, there was a significant decrease in the platelet count and fibrinogen levels (11). Thus, the autoanticoagulated state produced by our perfusion system and Fletcher's membrane oxygenator may differ.

The heparin activity that appeared in the blood of perfused dogs was not a contaminant washed from the tubing. No heparin activity was produced when saline, citrated whole blood, or nonanticoagulated blood was circulated in the perfusion circuit. It is possible, however, that a perturbation of the blood in the perfusion circuit leads to the appearance of the heparin activity. The nature of this perturbation requires further study. This effect of extracorporeal perfusion in the blood might not have been previously appreciated since the response to the perturbation would have been masked by administration of commercial heparin.

The results of this study may also be relevant to the study of endogenous heparin. Endogenous heparin may play a role in the regulation of fat metabolism and clotting (9,19). Abnormalities of endogenous heparin could be involved in the development of thrombosis and atherogenesis (9,19). Progress in this area has been hampered by the lack of a suitable model in which to study the endogenous heparin which may be present in the vascular space.

Endogenous heparin appears in dog blood during anaphylactic and peptone shock (7,27). This model has not been extensively used since the heparin was presumed to originate primarily from the canine liver. Hepatectomy prevented the dog blood from becoming incoagulable during anaphylaxis and reduced the amount of protamine needed to bring the clotting time back to the normal range. These results were interpreted as proof that heparin does not appear in the blood in canine anaphylaxis after hepatectomy (17). Contrary to this interpretation, we found from 1.7 to 4.8 U/ml of heparin activity present in the plasma of perfused eviscerated dogs with the hepatic artery tied without the development of incoagulable blood. Thus, the whole blood clotting time in dogs lacking a perfused liver may not be a reliable indicator of the presence or absence of heparin. Further studies are needed to better define the nature of this "heparin resistance".

There is increasing evidence for the existence of non-mast cell heparin (19). Administered heparin binds to endothelium and platelets and is taken up by the reticuloendothelial system (19). In addition, canine liver contains two heparin fractions determined by polyacrylamide gel electrophoresis and one of these fractions does not correlate with mast cell numbers (18). Since evisceration with ligation of the hepatic artery did not decrease the levels of plasma heparin activity compared to our normal perfused animals, it is unlikely that hepatic heparin release is the major cause of the

circulating heparin activity. It is possible that extracorporeal perfusion without added heparin may be a stable, non-shock model in which to study the non-mast cell heparin of the vascular space. For this reason, further studies are needed to define the source of this endogenous "heparin-like" material.

In conclusion, dogs perfused with an arteriovenous extracorporeal perfusion system develop an autoanticoagulated state related to the generation of plasma heparin activity. This system may serve as a useful model for future studies of endogenous heparin and the effects of extracorporeal perfusion on the blood.

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FIGURE LEGENDS

Figure 1. Changes in the whole blood clotting time during extracorporeal perfusion without administration of heparin. Blood samples were drawn from a brachial arterial cannula in anesthetized dogs during and after arteriovenous extracorporeal perfusion. N=6 dogs.

Bar = +SE.

Figure 2. Endogenous anticoagulation during extracorporeal perfusion: plasma heparin activity. Anesthetized dogs were placed on an arteriovenous extracorporeal perfusion apparatus without exogenous anticoagulants. Samples of blood drawn from the brachial artery were assayed for heparin activity using a thrombin clotting time of normal canine plasma. First, 0.05 ml of the citrated test plasma was added to 0.15 ml of normal citrated canine plasma at 37°C. Then 0.1 ml of IIa diluted in 0.1 ml CaCl_2 was added and the clotting time measured manually. A standard curve was constructed using commercial heparin (Upjohn; beef lung). Three hours of recovery followed 3 hours of perfusion.

Figure 3. Inhibition of Factor Xa or thrombin-initiated clotting of plasma by dog plasma. Clotting of bovine plasma was performed with either Factor Xa or thrombin as described in "Methods". Thrombin activity or Factor Xa activity was determined from thrombin or Factor Xa standard curves in the absence of inhibitor. Thrombin-initiated clotting (\ominus), Factor Xa-initiated clotting (\circ) and Factor Xa-initiated clotting using heparin (5 U/ml) (\bullet) in place of dog plasma as the inhibitor were studied.

Figure 4. The effect of plasma from a perfused dog on the inhibition of Factor Xa by antithrombin III or o-methyl-isourea derivatized antithrombin III (1.1 μM final concentration) at 37°C in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, 1 mg/ml bovine serum albumin. Factor Xa activity was measured as described in "Methods". In the top panel the reaction mixture also included: nothing (\bullet), 2 μl dog plasma (∇) and 1 μl dog plasma (∇). For the bottom panel Factor Xa (0.33 μM) was incubated with o-methylisourea derivatized antithrombin III (2.5 μM final concentration) in the presence of no addition (\square) or 2 μl dog plasma (∇). In addition, Factor Xa (0.33 μM) was incubated with 2 μl dog plasma in the absence of antithrombin III (∇).

Figure 5. Effect of plasma from a perfused dog on the inhibition of thrombin by antithrombin III. Thrombin (0.23 μM) was incubated with antithrombin III (1.1 μM final concentration) at 37°C in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, 1 mg/ml bovine serum albumin, with no addition (\square), 2 μl dog plasma (∇) and 1 μl dog plasma (∇). Thrombin was assayed as described in "Methods".

Figure 6. Heparin activity in eviscerated perfused dogs with the hepatic artery ligated. Anesthetized dogs were eviscerated with the stomach, intestines, spleen and pancreas removed and the hepatic artery ligated. The dogs were then placed on an arteriovenous extracorporeal perfusion apparatus without added heparin. Samples of blood drawn from the brachial arterial cannula were assayed for heparin activity by measuring the prolongation of the thrombin time of normal citrated canine plasma (see "Methods"). Two hours of perfusion was followed by two hours of recovery under anesthesia. N=6 dogs.

Figure 7. Whole blood clotting time during extracorporeal perfusion without added heparin in eviscerated dogs with the hepatic artery tied (see text Figure 7). Blood samples were drawn from the brachial arterial cannula of anesthetized dogs following evisceration and ligation of the hepatic artery during perfusion on the extracorporeal system. A modified Lee-White clotting time was used (see "Methods"). Note that these dogs did not attain whole blood clotting times greater than 24 hours despite the presence of plasma heparin activity (see Figure 7). N=6 dogs. Bar = \pm SE.

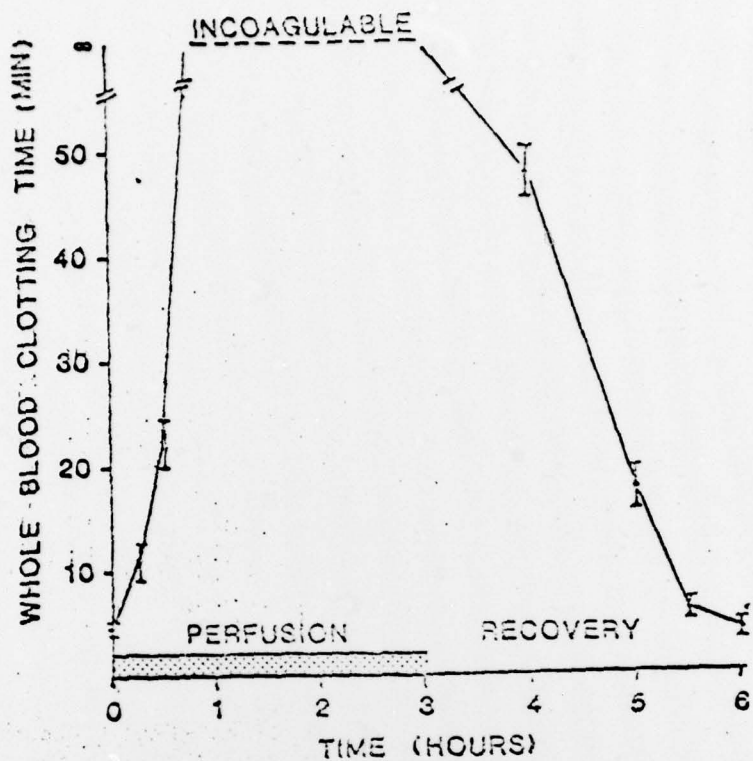
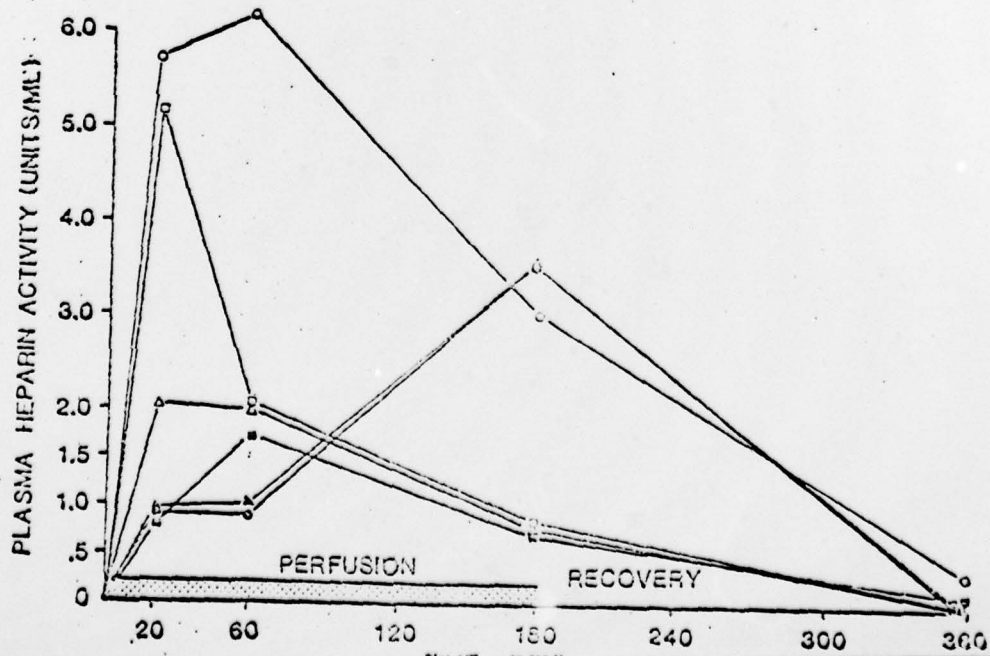


Fig. 1



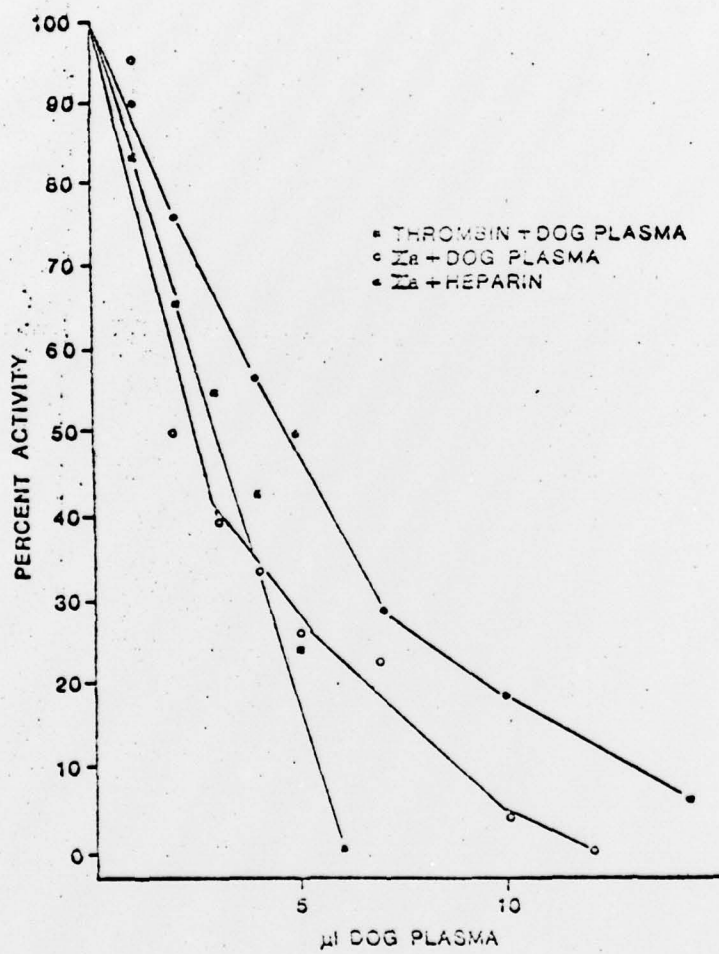


Fig. 3

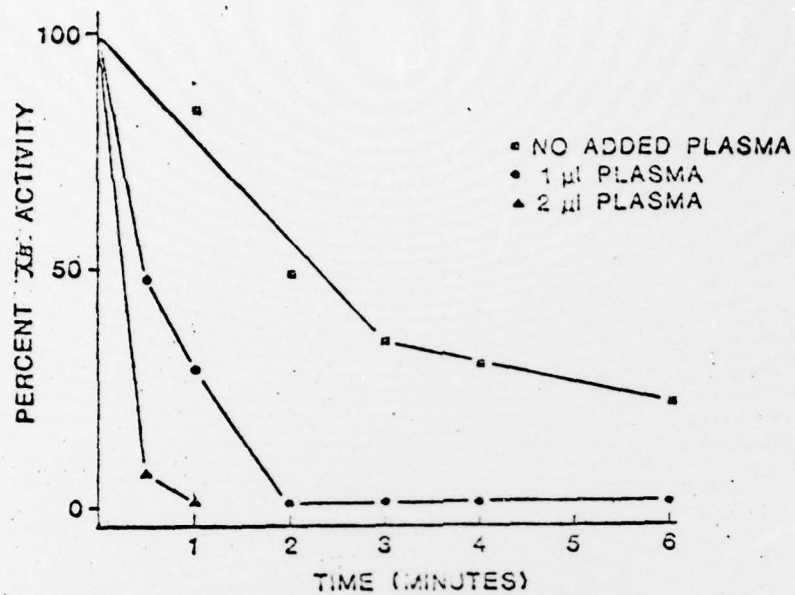


Fig. 4a

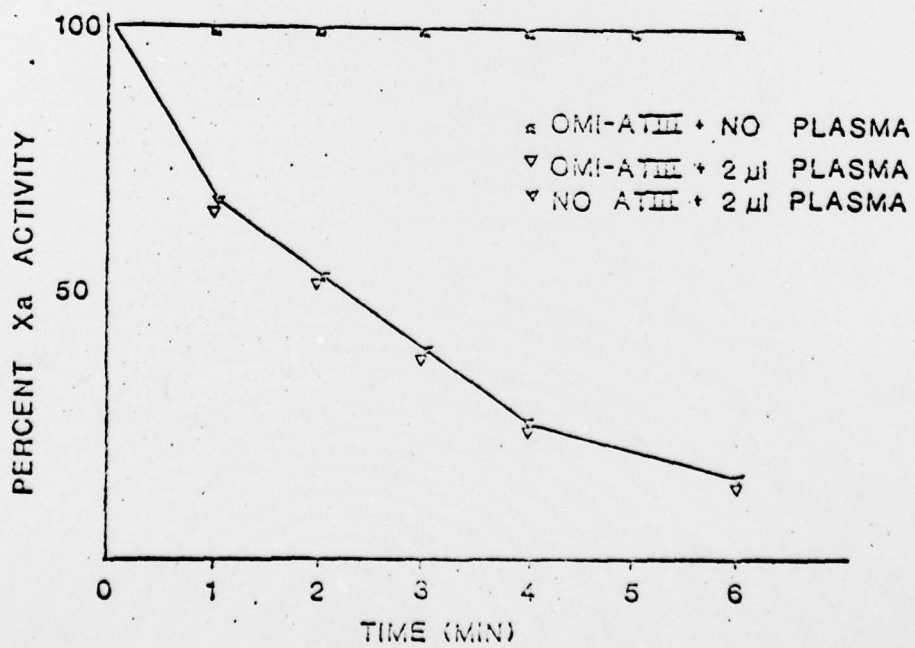


Fig. 4b

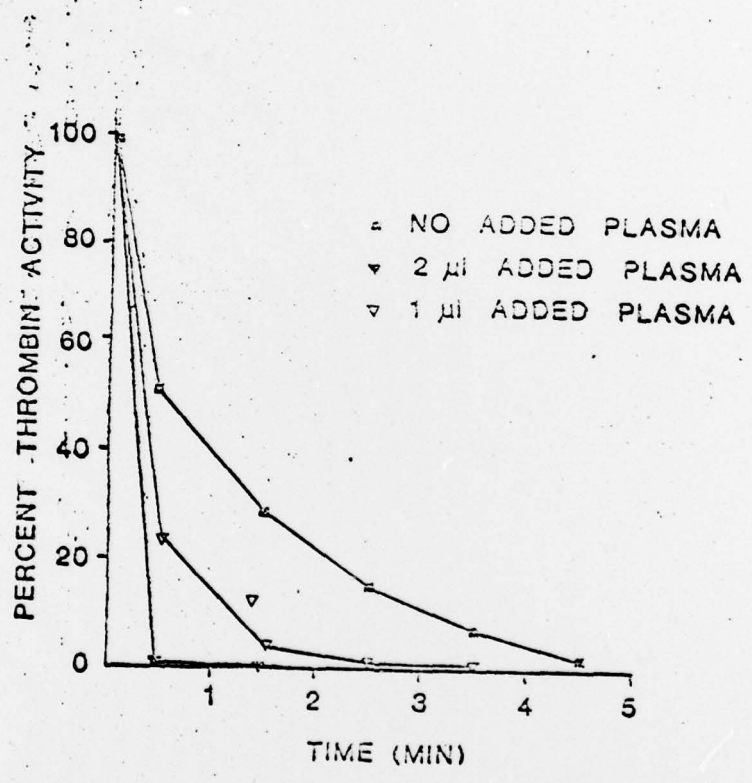


Fig. 5

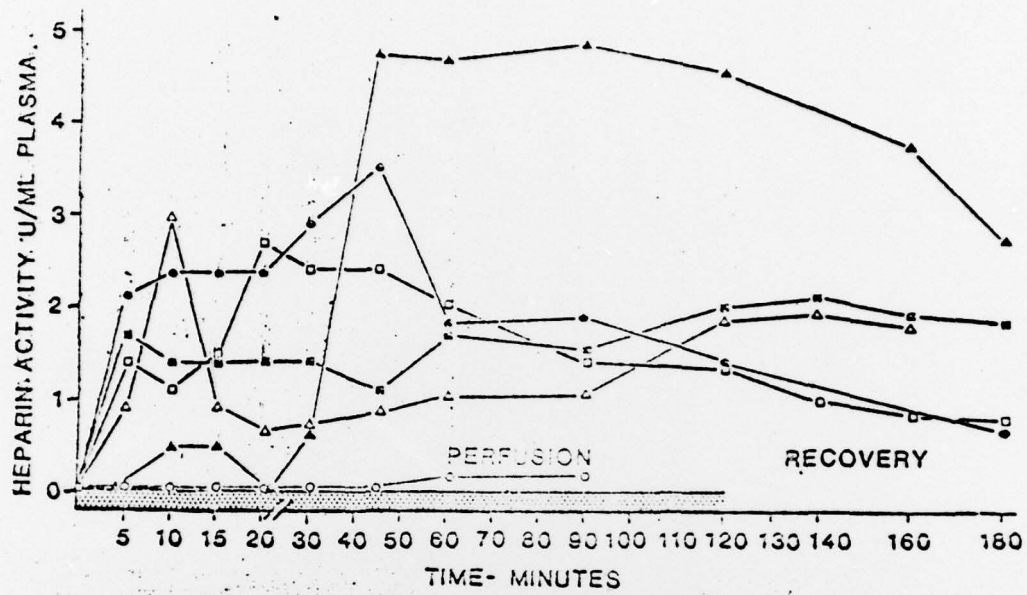
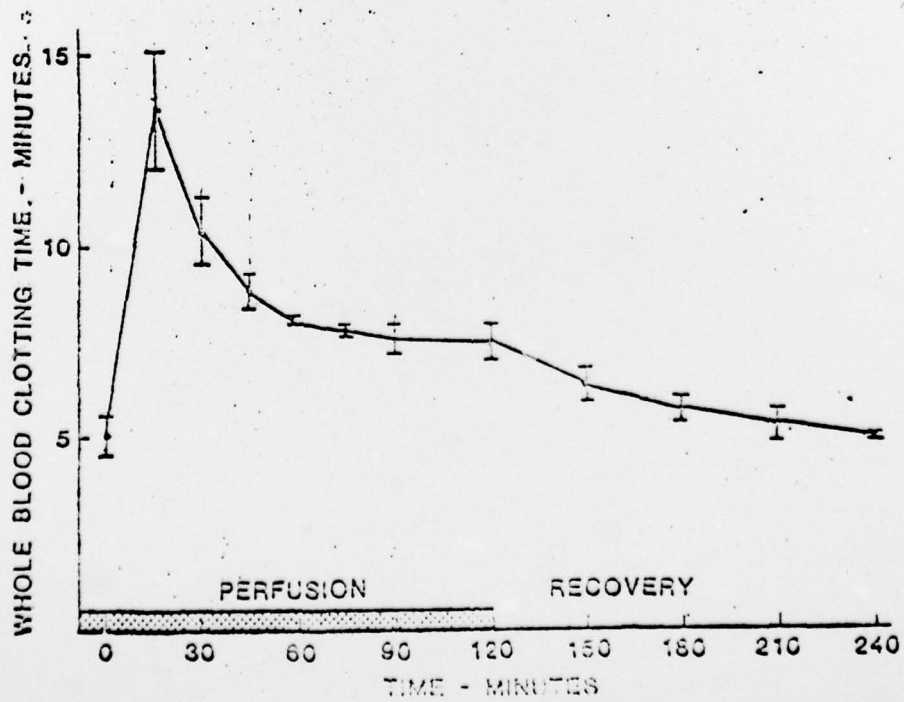


Fig. 6



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but not by o-methyl-isourea modified ATIII. Thrombin inhibition by ATIII was also stimulated. Six perfused dogs developed an equivalent of 0.98 to 6.15 U/ml of heparin activity determined by inhibition of thrombin-induced clotting of normal canine plasma. This activity was heat stable, adsorbed by BaSO₄, and neutralized by protamine. Infusion of protamine sulfate into two perfused dogs reversed the anticoagulant activity and brought the WBCT from greater than 24 hours to less than control. Five eviscerated dogs in which the hepatic artery was ligated developed peak plasma heparin activity of 3.2±1.0 U/ml. We conclude that dogs perfused on our extracorporeal perfusion system without added heparin develop an endogenous heparin activity in their plasma which is a major contributor to their autoanticoagulated state. In addition, this heparin activity may have an extra-hepatic origin.

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