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Ex vivo evaluation of a Taylor-Couette flow, immobilized heparinase I device for clinical application

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ABSTRACT **Efficient and safe heparin anticoagulation has remained a problem for continuous renal replacement therapies and intermittent hemodialysis for patients with acute renal failure. To make heparin therapy safer for the patient with acute renal failure at high risk of bleeding, we have proposed regional heparinization of the circuit via an immobilized heparinase I filter. This study tested a device based on Taylor-Couette flow and simultaneous separation/reaction for efficacy and safety of heparin removal in a sheep model. Heparinase I was immobilized onto agarose beads via cyanogen bromide activation. The device, referred to as a vortex flow plasmapheretic reactor, consisted of two concentric cylinders, a priming volume of 45 ml, a microporous membrane for plasma separation, and an outer compartment where the immobilized heparinase I was fluidized separately from the blood cells. Manual white cell and platelet counts, hematocrit, total protein, and fibrinogen assays were performed. Heparin levels were indirectly measured via whole-blood recalcification times (WBRTs). The vortex flow plasmapheretic reactor maintained significantly higher heparin levels in the extracorporeal circuit than in the sheep (device inlet WBRTs were 1.5 times the device outlet WBRTs) with no hemolysis. The reactor treatment did not effect any physiologically significant changes in complete blood cell counts, platelets, and protein levels for up to 2 hr of operation. Furthermore, gross necropsy and histopathology did not show any significant abnormalities in the kidney, liver, heart, brain, and spleen.**

The use of heparin, a common anticoagulant used during extracorporeal procedures, has been associated with a high incidence of hemorrhagic complications. (1, 2). Specifically, the patient suffering from acute renal failure in the intensive care unit is at a high risk of bleeding. For this reason, the management of heparin anticoagulation is of utmost importance for successful renal replacement therapy (3). One method to control blood heparin levels is regional heparinization, which can be an effective approach to minimizing exposure of the patient to heparin while maintaining adequate anticoagulation in the dialyzer and circuit tubing (4). During regional heparinization, heparin is infused into the extracorporeal circuit predialyzer, and neutralized with protamine postdialyzer. However, protamine, a highly basic protein that binds to heparin, has been reported to cause hypotension, vasodilation, pulmonary platelet accumulation, and bradycardia (5). In addition, the complexities related to protamine titration discourage its use. As a result, regional heparinization (though effective) is not widely used in the United States.

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Our laboratory has proposed the use of a medical reactor containing immobilized heparinase I for heparin neutralization (6). Heparinase I is an enzyme that specifically degrades heparin into nontoxic fragments and effectively eliminates its anticoagulant properties. An immobilized heparinase reactor could be placed postdialyzer to facilitate the practical aspects of regional heparinization and to eliminate the concerns associated with the use of protamine. However, the development of a safe and efficient bioreactor has been a significant challenge for the clinical application of immobilized heparinase I.

We are investigating a bioreactor design based on Taylor-Couette flow, plasmapheresis, and fluidization of agarose immobilized heparinase I. The design uses the principle of simultaneous separation and enzymatic reaction to minimize blood cell damage and the priming volume of the device. Taylor-Couette flow (also referred to as Taylor vortex flow) is present when the rotation rate of an inner cylinder within an annulus reaches a critical rotation rate (7). Taylor-Couette flow is characterized by the presence of periodic, counter rotating, flow cells termed Taylor vortices, which are depicted in Fig. 1. In filtration applications, Taylor vortices have been shown to significantly contribute to higher filtrate fluxes than those observed in hollow fiber devices (8). Previous studies using this device demonstrated that it could effect regional heparinization in saline as well as in human blood *in vitro* (9). The positive safety results from *in vitro* experiments with human blood supported further testing *ex vivo*. In addition, data from animal studies are a prerequisite to clinical trials in humans.

The objective of this work was to investigate the efficacy and safety of the reactor when used for regional heparinization in a sheep model. The sheep was the animal model of choice because of its comparable size to humans (no need to scale up), its similar hemodynamic characteristics to that of humans (10), and feasibility to compare the current data to that of previous research in this model (11). Clotting times were monitored prereactor and postreactor to indirectly measure heparin neutralization. Reactor safety was assessed by white blood cell and platelet counts, degree of hemolysis, and specific protein levels.

METHODS

Reactor Design. A schematic of the reactor, referred to as the vortex flow plasmapheretic reactor (VFPR), is shown in Fig. 2. The dimensions of the VFPR are summarized in Table 1. The details for the construction of the VFPR are presented

Abbreviations: VFPR, vortex flow plasmapheretic reactor; WBRT, whole blood recalcification time; ESEM, environmental scanning electron micrograph.

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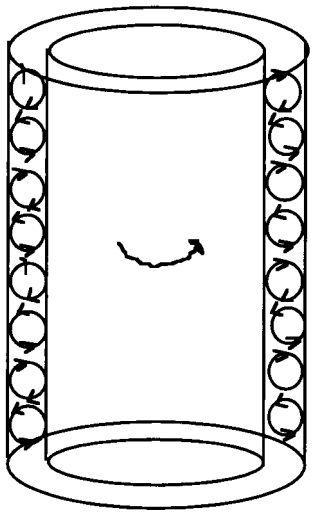

FIG. 1. Schematic of Taylor vortices. Taylor vortices are periodic secondary flows that develop once a critical rotation rate has been reached within the annulus of a rotating inner cylinder and a stationary outer cylinder.

elsewhere (9). The assembled device was gas-sterilized with ethylene oxide from Andersen (Oyster Bay, NY). Environmental scanning electron micrographs (ESEMs) of the polyester membrane and the agarose beads were performed at the Massachusetts Institute of Technology microscopy facilities. Chemical activation of porous agarose beads (6% crosslinked, 100 μm average wet bead diameter, from Bio-Rad) and the methods used to immobilize heparinase I are described elsewhere (9).

Measurement of Heparin in Blood. Clinically, the activated clotting time is the standard technique used to measure heparin activity in blood. However, the whole-blood recalcification time assay (WBRT) (12) was used because a faster determination of the anticoagulation status of the blood could

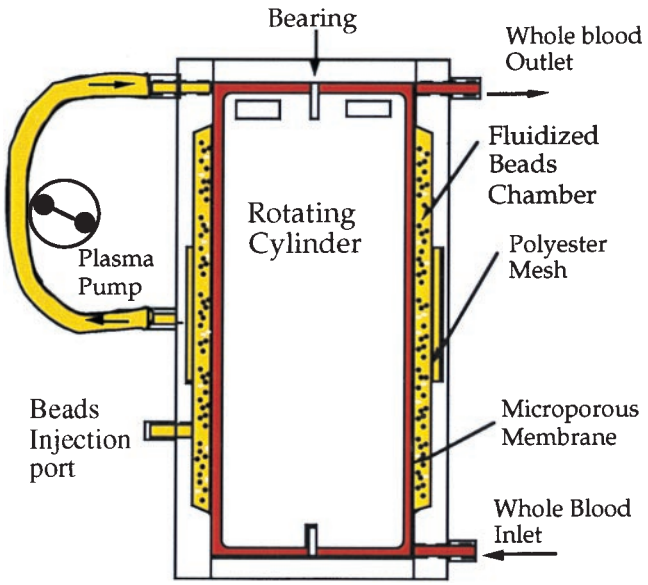

FIG. 2. Diagram of the VFPR. Flow is split between the whole blood path and the reactive volume via a microporous membrane. Undulations of the microporous membrane fluidize the agarose-immobilized heparinase I in the reactive chamber. The membrane undulations are a result of the flow dynamics induced by the rotation rate of the inner cylinder. The treated plasma is returned to the whole blood path where it is remixed with the cell components.

Table 1. Dimensions of the VFPR

Total reactor volume	125 cm^3
Reactor length	15 cm
Reaction chamber vol.	70 cm^3
r_i (inner cylinder)	2.70 cm
r_o (membrane)	2.87 cm
Gap width ($r_o - r_i$)	0.17 cm
Membrane length	11.0 cm

r_i, inner radius; r_o, outer radius. Republished from ref. 9 with permission.

be achieved *in situ* with a much smaller sample volume (200 μl vs. 2.0 ml). This assay is described elsewhere (9).

Animal Preparation and Regional Heparinization of the Circuit. The experimental protocol for the use of animals followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Massachusetts Institute of Technology Committee on Animal Care. Five sheep were used for the feasibility studies of the reactor. One animal was exposed to the reactor without agarose beads to assess whether the reactor materials and the complex fluid flow within the device would negatively affect the blood cells or the animal. The other four sheep were exposed to the agarose-immobilized heparinase I to simulate regional heparinization. A double lumen catheter (Medcomp, Harleysville, PA) was used to gain vascular access to the blood circulation of healthy male sheep (35–45 Kg). The catheter was inserted through the left internal jugular vein and guided into the right atrium of the heart. Blood flows of up to 200 ml/min were attained with no visible adverse effects to the animal. An extracorporeal procedure was performed up to four times on each animal depending on the patency of the catheter. A minimum of 2 days was allowed between each experiment performed on the same animal.

The experimental setup is shown in Fig. 3. The extracorporeal circuit consisted of a closed loop that included the animal, a blood pump, a plasma pump, a bubble trap, a thrombus trap, and the reactor. The bubble and thrombus traps help prevent bubbles and emboli, which may form during operation of the reactor, from entering into the animal. To incorporate and test the reactor in an extracorporeal circuit, standard pediatric dialysis tubing, generously donated by Children's Hospital (Boston, MA), was adapted with Tygon S-50 HL medical grade tubing and fittings obtained from VWR (Boston, MA) and Qosina Corporation (Edgewood, NY), respectively. The animal was awake for the duration of the procedure, which ranged from 1 to 2 hr. A special sling was used to prevent the animal from moving and injuring itself. Dialysis procedures may last up to 4 hr; however, extracorporeal circulation was maintained

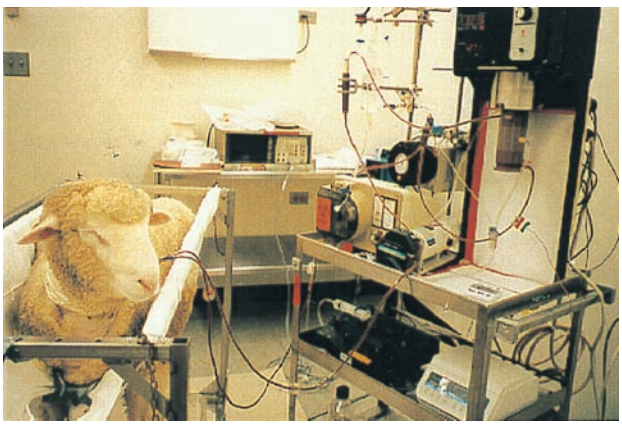

FIG. 3. Experimental setup for the *ex vivo* studies in sheep. The animal was conscious during the procedure but restrained within a specially designed sling.

for up to 2 hr to minimize any discomfort to the animal caused by the restraint. The total volume of the extracorporeal circuit was kept to a minimum to reduce hemodilution (under 150 ml).

The circuit initially was primed with normal saline obtained from Abbott. After all the air was purged, the immobilized enzyme (1:1 slurry in normal saline) was injected into the reactive volume. During enzyme infusion, the plasma pump flow rate and the inner cylinder rotation rate were set to 60 ml/min and 1,200 rpm, respectively. Before commencing extracorporeal circulation, an i.v. injection of heparin (5,000 United States Pharmacopoeia units) was administered to systemically heparinize the animal. The arterial and venous lines from the circuit were connected to their respective ports on the double lumen catheter. The saline used to prime the circuit was pumped back into the animal to compensate for the blood volume in the circuit. This procedure was tolerated well by the animal.

Regional heparinization of the circuit was implemented by infusing heparin into the arterial line at a rate of 13,000 United States Pharmacopoeia units/hr using a syringe infusion pump model Sage 361 (Sage Instruments, Boston, MA). This infusion rate, which is higher than that performed in humans, was necessary to prevent clotting in the circuit because sheep have platelet counts in the range of 200,000 to 800,000 per μl (13). The heparinized blood passed through the reactor, a venous thrombus trap, and back to the sheep. The blood flow rate in the extracorporeal circuit and plasma pump flow rate in the reactor were 150 and 55 ml/min, respectively. The rotation rate of the inner cylinder was 1,200 rpm. Blood samples were taken at the inlet and outlet of the reactor and assayed for heparin concentrations (WBRT assay) and other hematologic parameters. The pressure drops across the reactor inlet/outlet and across the microporous membrane were measured with two Tycos strain gauge pressure transducers (Tyco, Asheville, NC) to monitor any flow resistance.

The percent heparin effect removed by the reactor (also referred to as heparin conversion), %H, was calculated from:

$$\%\mathrm{H} = \left[1 - \left(\frac{\mathrm{WBRT_{outlet}} - \mathrm{WBRT_{baseline}}}{\mathrm{WBRT_{inlet}} - \mathrm{WBRT_{baseline}}}\right)\right] \times 100.$$

For a heparin filter to be clinically useful, heparin conversions in the range of 40% to 50% should be achieved. This target range is based on reactor inlet clotting times of 230–250 sec and reactor outlet clotting times of 180 $\pm$ 3 sec, according to the definition for percent heparin effect removed. These calculated clotting times assume a baseline or normal clotting time of 110 sec. The reactor was loaded with approximately 27 ml of agarose-immobilized heparinase with a specific activity of 18 units/ml of gel. The priming volume (whole-blood path) and cell-free volume (reaction chamber) of the reactor were 45 ml and 70 ml, respectively.

Hematology. Blood samples were collected from the reactor outlet into the appropriate vacutainer tubes (Becton Dickinson) at various time points. The following parameters were measured: white cell and platelet counts, hematocrit, fibrinogen, total protein (albumin + IgG), and plasma-free hemoglobin. The samples were analyzed manually for white cell counts, platelet counts, and hematocrit at the diagnostic laboratory of the Massachusetts Institute of Technology Division of Comparative Medicine. Fibrinogen and total protein were sent out for analysis at Quest Laboratories (Cambridge, MA). Cell counts and fibrinogen concentrations were normalized by the animal's baseline values and reported as percents. Plasma-free hemoglobin, which is an indicator of hemolysis, was measured in the laboratory by using a hemoglobin detection kit from Sigma.

Necropsy. The animals were euthanized by using an overdose of sodium pentobarbitol, which was injected directly into the catheter. A complete necropsy with a careful gross examination of all organs and tissues was performed. Tissue samples from the brain, kidney, spleen, liver, heart, and lung were taken for histological evaluation at the diagnostic laboratory of the Massachusetts Institute of Technology Division of Comparative Medicine.

RESULTS AND DISCUSSION

Reactor Safety. The results of the reactor safety control study, which exposed the sheep to the VFPR without the agarose beads, are shown in Fig. 4 *a* and *b*. The values reported are for the average of four experiments. There was no observed transient initial decrease in white cell counts (Fig. 4*a*, solid line) and platelet counts (Fig. 4*a*, dashed line) were within 10% of the initial values. Initial decreases in the white cell population usually are correlated with complement activation, which may promote sequestration of the white cells by the lungs (14). The concentrations of C3a and C5a (indicators of complement activation) were not measured for the *ex vivo* experiments because of the lack of an available standardized ovine complement assay. However, the absence of the white cell count drop within the first 30 min of operation suggests minimal complement activation. There is no substantial evidence of a negative clinical outcome in the form of increased morbidity or mortality among patients (14).

Fig. 4*b* shows the mean hematocrit (a measure of the volume percent of cells in plasma), which also was maintained within physiological levels (22–35% vol/vol). The maintenance of the hematocrit during the extracorporeal circulation is a positive sign because it is known that sheep can alter their hematocrit significantly when they are under stress (15). A significant decrease in hematocrit also could suggest internal bleeding within the animal or excessive red cell lysis (hemolysis). To further confirm the integrity of the red cells, plasma-free hemoglobin concentrations were measured before and after the extracorporeal procedure. The beginning and final (2 hr) plasma-free hemoglobin concentrations were 6 $\pm$ 0.6 and 7 $\pm$ 1.5 mg/deciliter, respectively. These low plasma-free hemo-

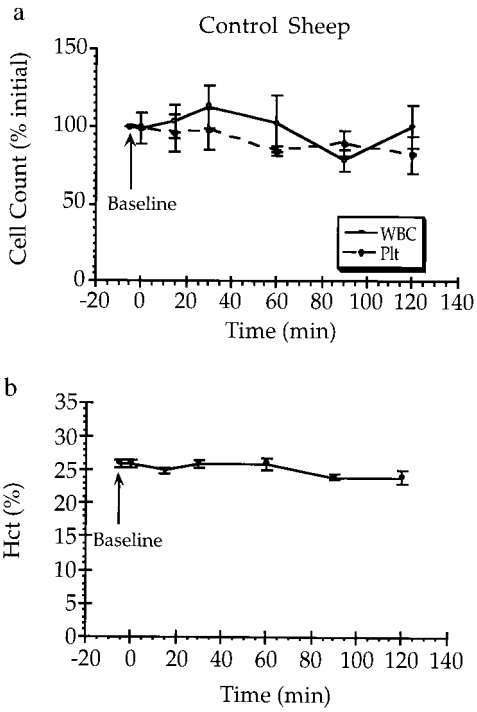

FIG. 4. Safety data for a control sheep. (*a*) White cell (solid line) and platelet (dashed line) count profiles were normalized with baseline values (9 $\pm$ 1 $10^3/\mu$l and 830 $\pm$ 90 $10^3/\mu$l, respectively). (*b*) Hematocrit. Mean $\pm$ SEM of four experiments.

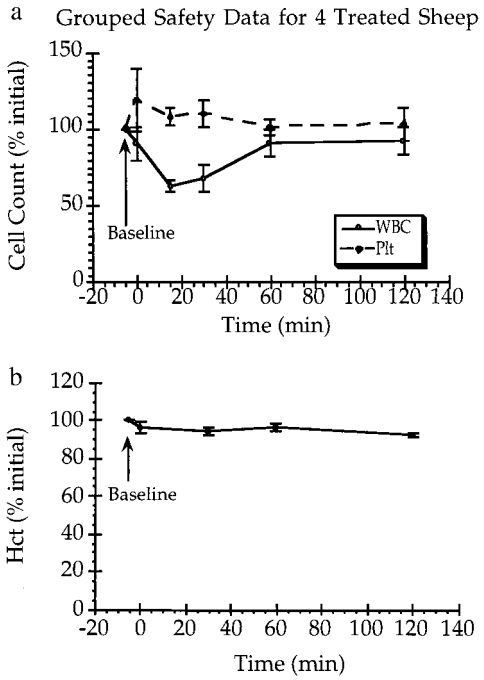

FIG. 5. Grouped safety data for a regionally heparinized sheep. (*a*) White cell and platelet count profiles were normalized with baseline values (7.0 ± 0.5 $10^3/\mu$l and 396 ± 37 $10^3/\mu$l, respectively). (*b*) Hematocrits also were normalized with baseline values (28 ± 0.8%). There was an initial drop in white cell count followed by a rebound to within 20% of initial cell count. Mean ± SEM of 11 experiments.

globin concentrations reflect a significant improvement in reactor design when compared with past hemolysis observations reported in the literature (11, 16). In these reports, previous immobilized heparinase devices based on fluidized agarose beads were tested in sheep and plasma-free hemoglobin concentrations of up to 90 mg/deciliter were found after 1 hr of operation. The data on the mean hematocrit together with the low levels of plasma-free hemoglobin generated during the experiment with the control sheep suggest a very small degree of hemolysis. No decrease in heparin anticoagulant activity was observed during the control experiments.

The reactor safety data for 11 regional heparinization studies performed on each of four animals were pooled, and the results are shown in Fig. 5. The white cell counts (Fig. 5*a*, solid line) seemed to follow the reported trends that are associated with complement activation. However, in most cases the white cell count rebounded to approximately 80% of the initial values within 1 hr and throughout the end of extracorporeal circulation. In addition, the white cell counts were within normal values before the beginning of subsequent experiments. Platelet counts (Fig. 5*a*, dashed line) were normal throughout the experiments. The maintenance of a normal platelet count during the experiments is another significant improvement over previous reactors, which depleted platelets down to 50% of the initial value after 1 hr of operation without

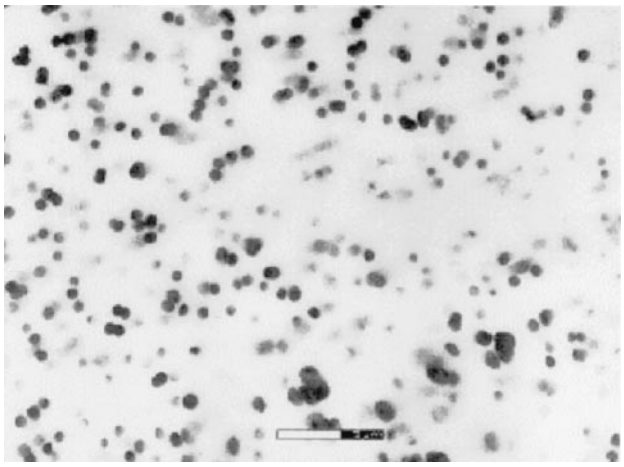

FIG. 6. ESEM of the polyester microporous membrane used to separate the cells from the agarose-immobilized enzyme within the reactor. The dark holes are the etched pores of the membrane, which were 1 μm in diameter. Sheep red cells range from 4 to 6 μm in diameter. (The bar represents 5 μm.)

a dialyzer (11). By the end of the regional heparinization procedure with the VFPR, plasma-free hemoglobin concentrations were below 15 mg/deciliter on average. Therefore, separation of the blood cells from the agarose-immobilized heparinase significantly minimized cell destruction.

The results for the total protein and fibrinogen measurements are shown in Table 2. After 1 hr of extracorporeal circulation, there was no significant change in the total protein for the one control or the four treated sheep. The mean of the normalized fibrinogen concentrations for three regionally heparinized animals and one control animal decreased to 94 ± 2% and 92 ± 3% of initial baseline concentrations, respectively, after 1 hr of extracorporeal circulation through the VFPR. The fact that fibrinogen levels decreased in both enzyme-exposed and control sheep suggests that the deheparinization process within the VFPR did not significantly augment clot formation as judged by this assay.

The VFPR did not experience any membrane clogging problems (as long as the undulations were present) for transmembrane plasma flow rates as high as 60 ml/min when judged by monitoring the pressure drop across the membrane. Fig. 6 shows the ESEM of a microporous polyester membrane that was recovered after a regional heparinization experiment and fixed in formalin. There were no adhered white cells or clots on the membrane surface. In addition, visual inspections of the reactors, at the end of each procedure, did not identify any macroscopic clots in the compartments of the device. To confirm the above findings, detailed hemostasis tests such as the prothrombin time and thrombin-antithrombin assays need to be performed. The small fibrinogen concentration changes observed in the current sheep studies (8%) compare favorably to similar measurements that were taken in a deheparinization system using a poly-L-lysine adsorbent device, which was tested in a bovine model (17). In those studies, fibrinogen levels were

Table 2. Total protein and fibrinogen values for experiments involving one control and four regionally heparinized sheep

	Total protein, g/deciliter		Fibrinogen, % initial	
	Control, 1 sheep 4 experiments	Reg. hep., 4 sheep 11 experiments	Control, 1 sheep 4 experiments	Reg. hep., 3 sheep 8 experiments
Before circulation	6.5 ± 0.2	6.1 ± 0.1	100	100
1 hr of circulation	6.2 ± 0.2	6.0 ± 0.2	92 ± 3	94 ± 2

Mean ± SEM. Reg. hep., regionally heparinized.

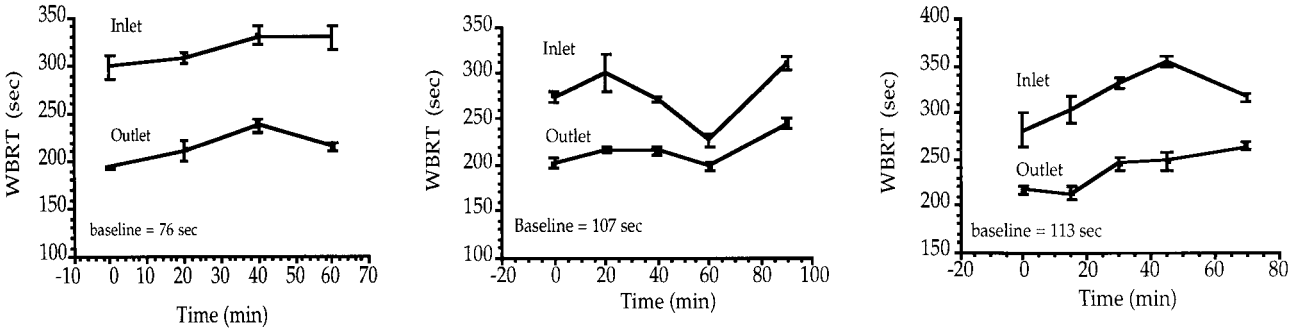

FIG. 7. Regional heparinization of a sheep demonstrating the capacity of the VFPR to maintain lower clotting times at the outlet of the device. Reactor inlet flow rate was 150 ml/min at 33°C. Plasma pump flow rate was 55 ml/min. Mean ± SEM of four measurements.

reduced by 21% at the end of 1 hr of operation using control (no adsorbent) and active devices.

The necropsy examinations, performed on all sheep, did not show any evidence of tissue damage caused by application of the VFPR. Tissue sections from the brain, kidney, spleen, liver, heart, and lung looked normal, which is encouraging given the large amount of heparin that was required to prevent clotting in the extracorporeal circuit. There were normal tissue healing responses associated with the placement of a catheter in the jugular vein.

Reactor Efficacy. The regional heparinization data for a representative set of experiments is shown in Fig. 7 *a–c*. As shown in the figure, the reactor could maintain a heparin concentration difference across the inlet and outlet, therefore effecting regional heparinization. The mean heparin conversion (%H) was calculated to be 42 ± 2, 36 ± 4, and 39 ± 4%, for the profiles shown in Fig. 7 *a-c*, respectively. The blood flow rate was 150 ml/min and the plasma flow rate was set to 55 ml/min. During the experiment, systemic WBRTs were similar to the WBRTs measured at the reactor outlet. Therefore, within experimental error, the heparin conversions achieved by the reactor are in the target range (see *Methods*), demonstrating the feasibility of safely achieving regional heparinization.

The reactor performance is explained as follows: because the average hematocrit for this particular animal was 27, the plasma flow through the reactor was 150*(1 − 0.27) = 110 ml/min. If the reactive chamber was 100% efficient, 50% of the plasma leaving the reactor would be cleared of heparin. However, mathematical modeling in our laboratory showed that for an enzyme loading of 20 ml of gel (16 units/ml gel) the predicted conversion in the reactive chamber would be 92.6% for a transmembrane flow rate of 55 ml/min (18). In addition, according to the model, 11% of the transmembrane flow bypasses the immobilized enzyme (49 ml/min of the transmembrane plasma flow effectively encounters the immobilized heparinase). By approximating the conversions in the reactor with the above values, the following conversion is expected:

$$\% \text{ Conversion} = 1 - \left[\frac{110 - 49 \times 0.926}{110}\right] \times 100 = 41\%.$$

This predicted conversion has a 5% error difference when compared with the mean conversion of 39 ± 2% obtained from the three regional heparinization experiments shown in Fig. 7 *a–c*.

During preliminary regional heparinization studies in sheep, a drop in conversion, usually on the order of 40% by the end of 90 min, was observed. The activity of the beads was tested *in vitro* with saline after the regional heparinization procedure in sheep. Approximately 64% of the enzymatic activity of the beads was recovered. Fig. 8 *a* and *b* shows ESEMs of immobilized heparinase agarose beads exposed to the sheep plasma within the reactor and plain agarose beads in saline, respectively. There was a significant difference in surface morphology between the two samples probably because of protein deposition or micro clots. Specifically, there were globular patches throughout the surface of the beads.

After taking the observed specific activity drop into consideration, the remaining enzymatic activity on the agarose beads should have been enough (in the absence of significant external mass transfer limitations) to maintain the desired heparin conversions in the circuit. External mass transfer limitations are minimized as long as adequate bead fluidization is maintained within the reactor. It is possible that fluid bypassing through the reactive compartment may be an issue for the current reactor configuration. It also was observed

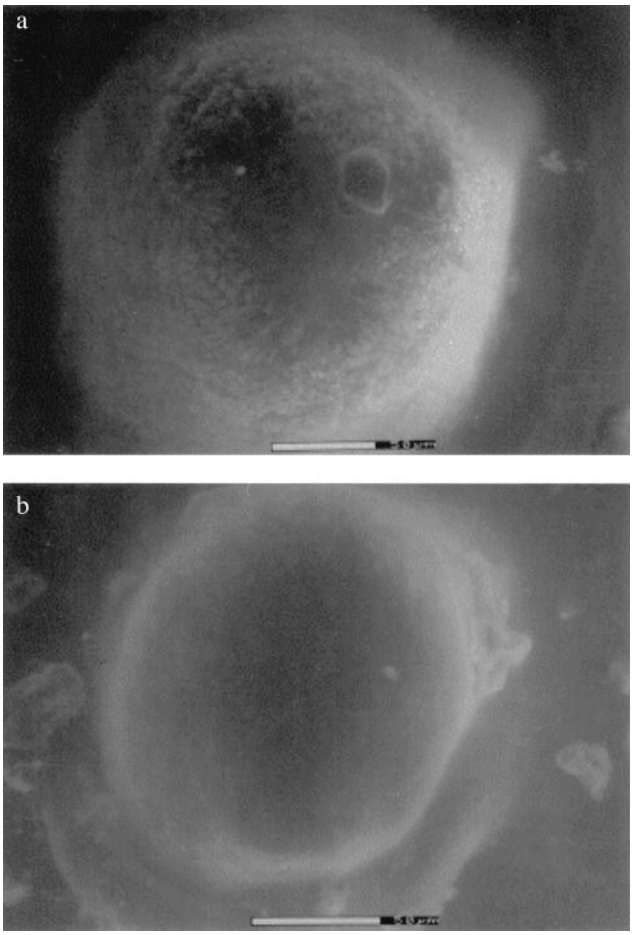

FIG. 8. (*a*) ESEM of an agarose-immobilized heparinase bead after a regional heparinization study in the VFPR. The surface of the bead is covered with what may be a proteinaceous material. (*b*) ESEM of a control agarose-immobilized heparinase bead that was exposed to saline. (The bar represents 50 μm.)

during the regional heparinization experiments that within 30 min of reactor operation a small concentration gradient of agarose-immobilized heparinase was visible in the reactive chamber. The concentration of fluidized beads appeared higher at the bottom end of the chamber than at the top. Therefore the observed decrease in enzymatic activity could be attributed to a mechanical phenomenon (reactor induced) or to a physico-chemical process that takes place within the beads. Taking all of the observations into account, it is plausible that the following situations may be occurring. (*i*) Deposition of plasma proteins onto the agarose-immobilized heparinase increases the specific density of the agarose beads, effectively increasing their settling velocity. Although the beads are fluidized within circumferencial flow in the reactive chamber, the probability for bypass flow is significantly increased because a larger amount of the beads migrate to the bottom half of the reactive chamber (mechanical phenomenon). (*ii*) The high specific activity on the beads effectively creates a heparin-free zone within the inner pores of the beads. This zone may promote the formation of fibrin meshes (microclots) within the pores of the beads. These microclots block or inhibit the diffusion pathway of the heparin-antithrombin complex, making it inaccessible to the enzyme (physico-chemical process). This case would significantly reduce the biological enzymatic activity of the immobilized enzyme, especially at the low heparin concentrations used in clinical applications.

This study has shown that the reactor design based on Taylor-Couette flow, simultaneous separation and reaction, and agarose fluidization within circumferencial flow could safely maintain a heparin concentration difference within the extracorporeal circuit. Reactor-induced hemolysis was negligible, and blood cell counts were within normal range by the end of the procedure. These data support the feasibility of using this medical device to achieve regional heparinization. With further optimization and testing, the reactor potentially could be used for regional heparinization in humans to make heparin anticoagulation safer for the acutely ill patient in the intensive care unit.

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