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The Use of Immobilized Protamine in Removing Heparin and Preventing Protamine-induced Complications during Extracorporeal Blood Circulation

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Heparin, currently used in extracorporeal blood circulation procedures, may lead to hemorrhagic complications. Protamine, used for reversal of heparin-induced anticoagulation at the end of such procedures, can cause adverse hemodynamic responses. To prevent both types of complications, we have developed a reactor device containing immobilized protamine (*i.e.*, a protamine bio-reactor) that can be placed at the distal end of the circuit, thus providing simultaneous extracorporeal heparin removal and protamine treatment. In preliminary *in vivo* studies involving dogs at a blood flow of 100 ml/min, the bio-reactor removed about 50% of the administered dose of heparin (*i.e.*, 100 units/kg) in 10 min. While rapid injection of protamine in dogs anticoagulated with heparin produced a transient and significant ($P < 0.005$) decreases in systemic arterial blood pressure (−39.5 ± 9.2 mmHg), cardiac output (−1.59 ± 0.23 L/min), and mixed venous oxygen saturation (−7.5 ± 1.3%) and increases in pulmonary artery systolic (+12.7 ± 4.4 mmHg) and diastolic pressures (+10.0 ± 3.6 mmHg), the use of the protamine bio-reactor did not elicit any statistically significant change in any of the variables measured. Hemolysis was not significant, as reflected by a statistically insignificant change of the animals' red blood cell counts, hematocrits, and total hemoglobin values. In addition, hemolytic complement was found to be reduced only by 10% in animals with the protamine bio-reactor, whereas it was reduced rapidly by 20% in animals receiving intravenous protamine administration and progressively by 20% in control animals with a sham reactor that contained no protamine. Furthermore, the use of the protamine bio-reactor also significantly reduced the protamine-induced transient thrombocytopenic and granulocytopenic responses. The white blood cell counts and platelet counts decreased to 87.7 ± 7.5 and 83.3 ± 5.0% of baseline, respectively, in dogs with the protamine bio-reactor compared to 35.5 ± 14.3 and 32.1 ± 8.1% of baseline in dogs receiving intravenous protamine. The protamine bio-reactor may provide a unique means to simultaneously control both heparin- and protamine-induced complications. (Key words: Blood, anticoagulants: heparin. Blood, coagulation: protamine; complement activation. Surgery, cardiac: cardiopulmonary bypass.)

EXTRACORPOREAL BLOOD CIRCULATION (ECBC) is used in clinical situations such as renal dialysis, cardiothoracic surgery, organ transplantation, extracorporeal membrane oxygenation, and the implantation of artificial organs. It is estimated that approximately 20,000,000 procedures requiring ECBC are performed each year.[1,**]

In all of these applications, high systemic concentrations of heparin are required to prevent clotting, which may be initiated when blood comes into contact with the ECBC device (fig. 1A). However, the use of heparin may be associated with undesired bleeding,[2,3] and therefore protamine sulfate usually is administered to reverse the anticoagulant effect of heparin.[4–6] Unfortunately, the administration of protamine may cause adverse hemodynamic side effects.[7–11] Protamine reactions range from mild hypotension[8,9] to severe or ultimately fatal cardiovascular collapse.[10,11] A number of approaches have been attempted to modify the response to protamine. They include altering the route and rate of protamine administration,[8,12,13] pretreating patients with small doses of protamine,[13,14] and using agents such as antihistamines and steroids.[7] However, there still is no good means of controlling the bleeding risks associated with the systemic use of heparin or the hemodynamic effects resulting from the use of protamine for heparin neutralization.

We propose an approach both to remove heparin and to prevent protamine-induced complications.†† The approach consists of placing a blood-compatible reactor device containing immobilized protamine (defined as a protamine bio-reactor) at the distal end of the ECBC circuit (fig. 1B). The protamine bio-reactor binds and selectively removes heparin after heparin induces anticoagulation in the extracorporeal device and before it is returned to the patient. In addition, the protamine bio-reactor restricts the heparin–protamine complexes from returning to the patient. Since the response to protamine most probably is related to the circulating heparin–prot-

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The protamine bio-reactor technology discussed in this paper is covered by an issued United States patent (patent 4,800,016) owned by the University of Michigan, with Victor C. Yang as the inventor.

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** Kambic HE, Murabayashi S, Nose Y: Biomaterials in artificial organs. Chemical and Engineering News April 14 issue, pp 31–48, 1986.

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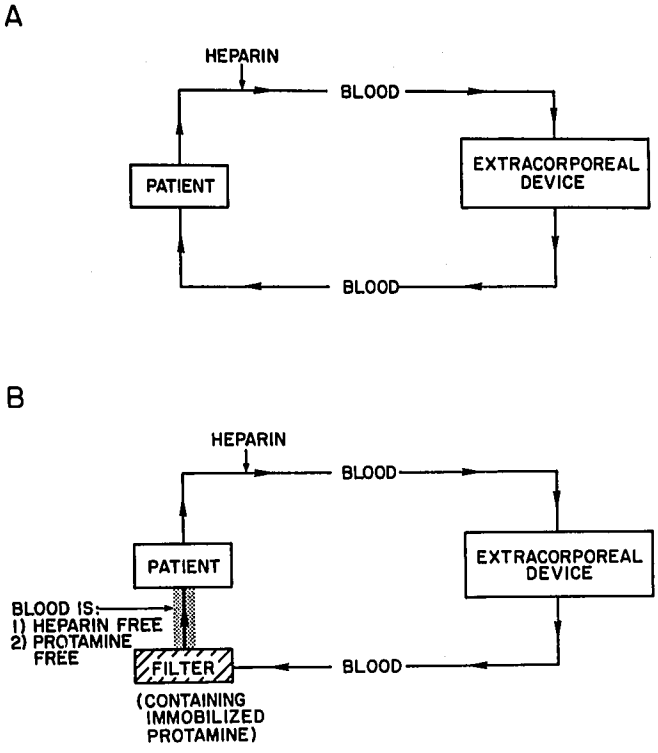

FIG. 1. *A:* Current extracorporeal circulation. *B:* Proposed extracorporeal blood circulation.

amine complexes that secondarily initiate the generation of eicosanoid, particularly thromboxane,[15,16] the use of this device theoretically would minimize the adverse effects of protamine.

The primary objective of this study was to demonstrate the feasibility of using a protamine bio-reactor to remove heparin and prevent protamine-induced complications during an ECBC procedure. A femoral arteriovenous bypass circuit was selected as the experimental model for this *in vivo* feasibility study because it mimicked the simplest clinical situation for extracorporeal circulation. Presented here are results of preliminary canine studies.

Materials and Methods

Assays

Protamine concentration was measured according to the method of Bradford.[17] This method of protein determination is based on the binding of Coomassie Brilliant Blue G-250 to protein, which results in a shift in the absorption maximum of the dye from 465 to 595 nm, and has been found very sensitive for measuring protamine concentrations in the range of 1–5 μg/ml. Plasma heparin activity was determined by the activated partial thromboplastin time (aPTT).[18] This clotting assay is based on the activation of the intrinsic system through factor XII, using the activated Cephaloplastin reagent (Dade Diagnostics, Aguada, Puerto Rico). The assay was found linear with heparin activity in the range of 0.0–0.7 units/ml. A standard curve was constructed for each animal. Samples with heparin activity outside the linear range of the standard curve were diluted with normal plasma from the same animal. To validate the clotting results, plasma heparin activity was also determined by the thrombin time (TT) clotting assay.[18] This assay measures thrombin-mediated conversion of fibrinogen to fibrin using the Fibrindex human thrombin reagent (Ortho Diagnostics, Raritan, NJ). All of these clotting tests were performed on a Fibrometer manufactured by BBL Microbiology Systems (Cockeysville, MD). Complement activation was measured by the hemolytic complement assay (the CH_{50} assay)[19] using antibody-coated sheep erythrocytes.

Fabrication of the Reactor Device

Regenerated cellulose hollow fibers were obtained by disassembling a Travenol model 1500 CF hemodialyzer (Travenol Laboratories, Deerfield, IL). Each fiber has an internal diameter of 200 μm and a wall thickness of 10 μm and is made of Cuprophane membrane with a nominal molecular weight cut-off of 10,000 D. The fibers were cut to a length of 15 cm. Approximately 1,800 of the cut fibers (a dry weight of about 6 g) were collected and tied together at each end with a Teflon film to form a bundle. The bundle was then housed in a piece of tygon tubing (inner diameter ÷ 2 cm), which was fitted with a molded connector at each end. To each of the connectors, a piece of tygon tubing (inner diameter ÷ 0.5 cm and length 6 cm) containing a three-way valve was attached to allow for the collection of blood samples. All junctions were sealed with epoxy, and the epoxy was cured at room temperature until it hardened. The fiber bundle thus prepared possesses the hemodynamic characteristics mimicking those of the clinically used hemodialyzer and has a total inner surface area of 0.18 m^2 for protamine immobilization. Figure 2 is a photograph of this fabricated bundle device.

Protamine Immobilization

Protamine sulfate was immobilized onto the inner walls of the cellulose hollow fibers according to a modified procedure of March *et al.*[20] The prepared bundle of fibers was circulated with 1 M sodium carbonate solution for 5 min. After the circulation, the bundle was placed in a fume hood and circulated with 20 ml cyanogen bromide (CNBr) in acetonitrile (1 g CNBr per ml acetonitrile) for 5 min. The CNBr-activated bundle was then washed with 300 ml distilled water, 200 ml of 1 mM HCl solution, and 200 ml 0.1 M $NaHCO_3$ buffer (*p*H 8.3) containing 0.5 M

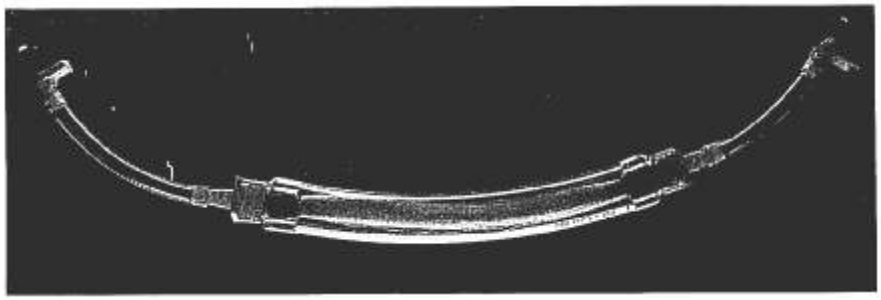

FIG. 2. The fabricated protamine bio-reactor.

NaCl. Immediately after the washing steps, 20 ml protamine solution (10 mg/ml, Eli Lilly & Co., Indianapolis, IN) containing 0.1 M $NaHCO_3$ and 0.5 M NaCl at *p*H 8.3 was circulated through the bundle at a flow rate of 5 ml/min over a period of 3 h at room temperature. The amount of protamine immobilized on the bundle was determined by measuring the difference of the protamine concentration in the solution before and after the immobilization procedure. The bundle thus prepared, which contains immobilized protamine, was defined as the "protamine bio-reactor".

In Vivo Experiments

Healthy female mongrel dogs, weighing 20–30 kg, were selected for the study. Each animal was anesthetized with sodium pentobarbital (30 mg/kg), and the lungs were mechanically ventilated to maintain physiologic blood gases. Hydration was maintained by administration of lactated Ringer's solution (in 20-ml/kg bolus followed by a 10-ml $\cdot kg^{-1} \cdot h^{-1}$ infusion). The femoral artery and the femoral vein of the animal were cannulated, and to the cannulas a hollow fiber bundle was attached to allow blood to flow from the artery through the filter and into the vein. Blood flow through the fiber bundle was measured with an electromagnetic square-wave flow meter (Carolina Instruments, King, NC) with the flow probe placed on the femoral vein. A venous thrombus trap (Baxter Healthcare Corporation, Round Lake, IL) consisting of an interior trap made of nylon with 200-μm meshes was placed at the effluent site of the bundle to prevent clots from entering the animal. Heparin (1,000 units/ml, Elkins-Sinn, Cherry Hill, NJ) was administered intravenously at a dose of 150 units/kg body weight.

Twenty minutes after heparin administration, the two valves on the hollow fiber bundle were opened to allow blood to circulate through the bundle. The time of opening the valves was defined as "time zero." Blood samples taken immediately before the opening of the valves were used as the baseline samples. An oximetric catheter with an optical fiber (Oximetrix, Mountain View, CA) was placed into the pulmonary artery for continuous determination of mixed venous oxygen saturation ($S\bar{v}_{O_2}$) and pulmonary artery systolic and diastolic pressures and for intermittent measurements of cardiac output using a thermodilution technique. Catheters were also inserted into the left carotid artery and jugular vein for monitoring systemic arterial blood pressure and blood withdrawal.

Twenty dogs were used for all *in vivo* studies. The animals were distributed into three groups (groups 1–3). Group 1 consisted of six dogs used as controls. In this group of dogs, a sham reactor containing no protamine was used, and the dogs were not given any protamine intravenously. Group 2 consisted of eight dogs used for the testing of the protamine bio-reactor. In this group the extracorporeal circuit was incorporated with a protamine bio-reactor that contained 60–70 mg immobilized protamine, and no additional protamine was administered. Group 3 consisted of six dogs used for testing the response to direct intravenous administration of protamine. In this group the extracorporeal circuit was incorporated with a sham reactor containing no protamine, and protamine was administered at time zero as a bolus dose (1 mg protamine per 100 units of heparin) over a period of 5 s.

The blood activated clotting time (ACT) in all animals was monitored periodically with a Hemocron (International Technidyne Corp., Metuchen, NJ). The plasma heparin activity was also determined using the aPTT and TT assays. Hemodynamic changes in the animal were monitored every minute for 5 min, and then at 10, 20, 30, and 60 min after the fiber bundle was opened. Blood samples were collected for all of the dogs from the arterial tubing of the bundle at 3, 10, 20, 30, and 60 min after the fiber bundle was opened. Samples were drawn into Vacutainer tubes containing EDTA (Becton Dickinson, Rockville, MD) for measurements of hematocrit, total blood hemoglobin, and complete blood cell counts, and into vacutainer tubes containing sodium citrate for determinations of the heparin activity and the activation of the complement system.

The use of vertebrate animals for this study was approved by the University of Michigan on Use and Care of Animals (approval 2683A). The University of Michigan is fully accredited by the American Association for Accreditation of Laboratory Animal Care, and the animal

care and use program conforms to the standards in The Guide for the Care and Use of Laboratory Animals.‡‡

Data Analysis

Data are presented as the mean ± the standard deviation (SD). Statistical comparisons over time and between animal groups were conducted by analysis of variance for repeated measures, followed by Duncan's multiple comparison test if the analysis of variance resulted in a value of $P < 0.05$.[21] In the complement studies, data obtained at various time intervals were statistically compared with those of time zero to identify if the change from baseline was significant.

Results

The rate of disappearance of heparin anticoagulant activity in the three animal groups is shown in fig. 3. In animals receiving intravenous protamine there was a rapid heparin reversal. Three minutes after protamine administration, the blood heparin activity, as measured by the aPTT assay, was already decreased to the minimum. Although the ACT assay indicated a full reversal of heparin anticoagulant activity (the ACT values had returned to baseline), the more sensitive aPTT assay nevertheless revealed that nearly 10% of the initial heparin activity remained.

In contrast, heparin removal by the protamine bio-reactor appeared to follow a pattern consistent with a slower, regional-type, two-compartment kinetic model. A rapid removal, presumably due to the immediate binding of heparin to the bio-reactor, occurred within the first 5 min after the opening of the protamine bio-reactor. This rapid heparin removal was followed by a second, much slower but relatively linear removal rate over 60 min, after which the remaining heparin activity had declined to a value close to that observed in animals receiving intravenous protamine administration. The TT assay gave similar results (data not shown). It should be noted that part of the heparin disappearance during the use of the protamine bio-reactor was attributed to the natural metabolism of heparin, as reflected by the time-dependent decay in heparin activity in the animals not receiving any protamine treatment (fig. 3). Blood continued to flow in an unrestricted fashion in the protamine bio-reactor during the course of the experiment (60 min). However, the flow slowed slightly (from an average of 100 ml/min to about 85 ml/min) near the end of the experiment (about 75 min).

‡‡ The National Institutes of Health: The Guide for the Care and Use of Laboratory Animals. National Institutes of Health publication no. 80-23, revised 1985.

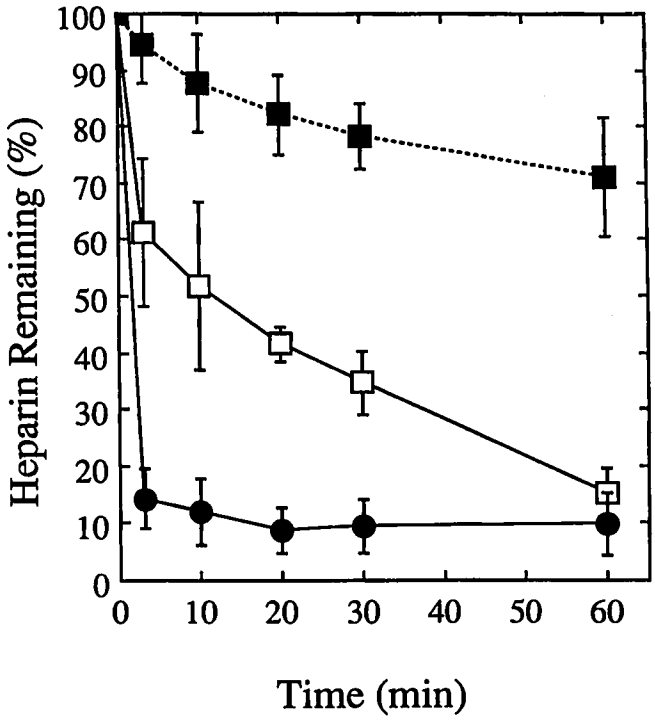

FIG. 3. *In vivo* heparin removal. Heparin activity was determined by the aPTT heparin clotting assay. The data were normalized to 0% activity before heparin administration and 100% activity after heparin administration. Filled squares = group 1 animals (*i.e.*, control dogs that received no protamine treatment); open squares = group 2 animals (*i.e.*, dogs with the protamine bio-reactor); and circles = group 3 animals (*i.e.*, dogs that received intravenous administration of protamine). The data are presented as the mean ± SD.

At the end of the experiments, all fiber bundles were removed and rinsed with saline solution. A sample of the fibers were then collected, cleaved with a single razor cut to expose the lumen of the fibers, and examined with a scanning electron microscope for the formation or deposition of blood clots on the surface of the lumen. Fibrin clots, some of them apparently intertwined with platelets, were seen in almost all of the sham reactors obtained from the group 3 animals, which received intravenous protamine for heparin reversal. They were detected only sparingly, however, in one third of the protamine bio-reactors obtained from the group 2 animals. Since none of the clots was observed in the thrombus trap located at the effluent site of the fiber bundle, formation of clots appeared to occur only within the bundle and on the fiber surface.

As shown in figure 4, the control dogs with untreated sham reactors did not display any clinically significant hemodynamic changes with time. None of the changes was statistically significant when the data obtained at each time interval were compared to those at time zero. However, dogs receiving intravenous protamine administration

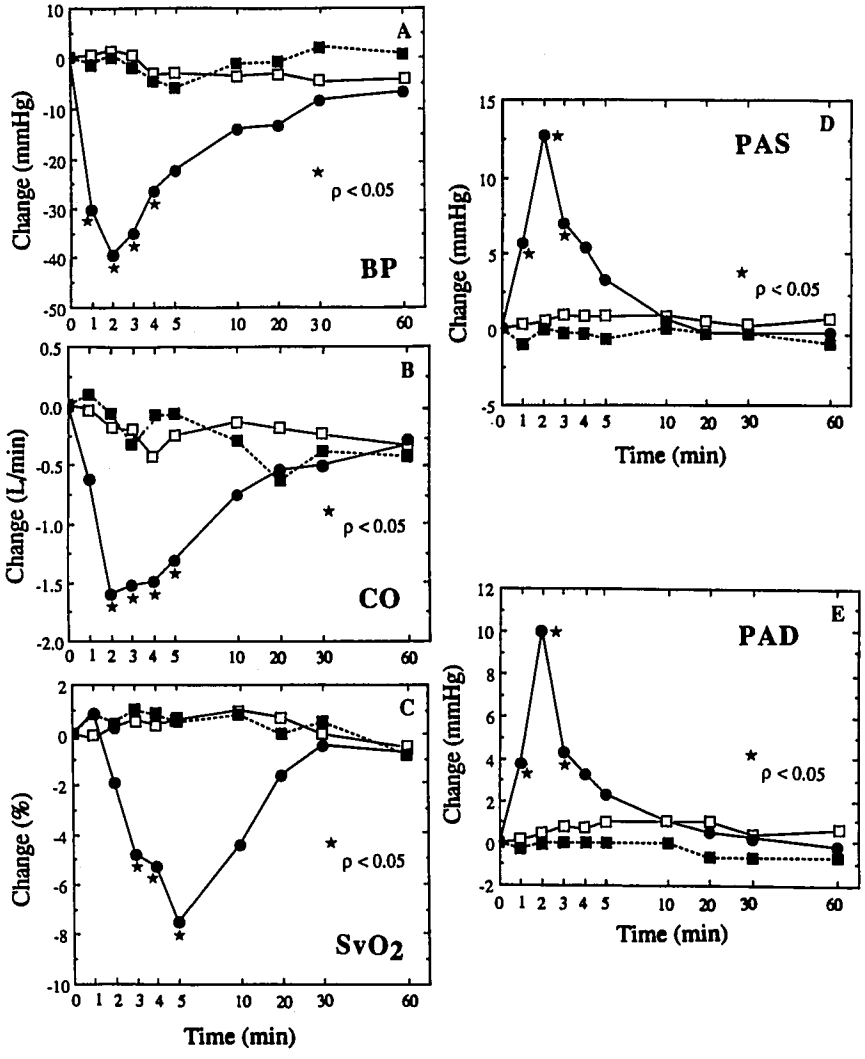

FIG. 4. Hemodynamic changes in dogs. Hemodynamic parameters measured include (*A*) systemic arterial blood pressure (BP); (*B*) cardiac output (CO); (*C*) mixed venous oxygen saturation ($S\bar{v}_{O_2}$); (*D*) pulmonary artery systolic pressure (PAS); and (*E*) pulmonary artery diastolic pressure (PAD). The experimental procedures are described in detail in the Materials and Methods section. Filled squares = group 1 animals (*i.e.*, control dogs that received no protamine treatment); open squares = group 2 animals (*i.e.*, dogs with the protamine bio-reactor); and circles = group 3 animals (*i.e.*, dogs that received intravenous administration of protamine). To avoid clustering in the figure, data points were represent only by the mean values. $*P < 0.05$ from the control group.

demonstrated typical responses to protamine. Marked changes were observed in all of the hemodynamic indices measured; these included decreases in systemic arterial blood pressure (−39.5 ± 9.2 mmHg, fig. 4A), cardiac output (−1.59 ± 0.23 l/min, fig. 4B), and mixed venous oxygen saturation (−7.5 ± 1.3%, fig. 4C), in conjunction with progressive increases in pulmonary artery systolic (+12.7 ± 4.4 mmHg, fig. 4D) and diastolic pressures (+10.0 ± 3.6 mmHg, fig. 4E). The changes were statistically significant ($P < 0.05$) at 1 min (except for cardiac output and $S\bar{v}_{O_2}$), 2 min (except for $S\bar{v}_{O_2}$), and 3 and 4 min (except for pulmonary artery systolic pressure) when compared with the control animals; the maximum changes occurred at the 2-min interval (except for $S\bar{v}_{O_2}$, which showed a maximum change at 5 min). All of the hemodynamic responses were transient and had returned to baseline 30 min after protamine injection.

In contrast, use of the protamine bio-reactor did not elicit any protamine-related hemodynamic responses (fig. 4). None of the changes observed at any time interval was statistically significant, when the data were compared to those at time zero. There were also no statistically significant differences in all of the parameters measured, when the changes were compared with those in the control animals. It should be pointed out that the relatively large decrease in cardiac output (−0.5 l/min) observed in the control group at the 20-min interval may be attributed to an additional, supplemental administration of pentobarbital to certain control animals.

The effects of the protamine bio-reactor on red blood cell counts, white blood cell counts, platelet counts, hematocrit, and total blood hemoglobin were examined. As shown in fig. 5A, the red blood cell counts in all animals, including both the control and experimental dogs, remained essentially unchanged throughout the experiment. Any change in red blood cell counts among different animal groups was not statistically significant, and the change with time also was not statistically significant.

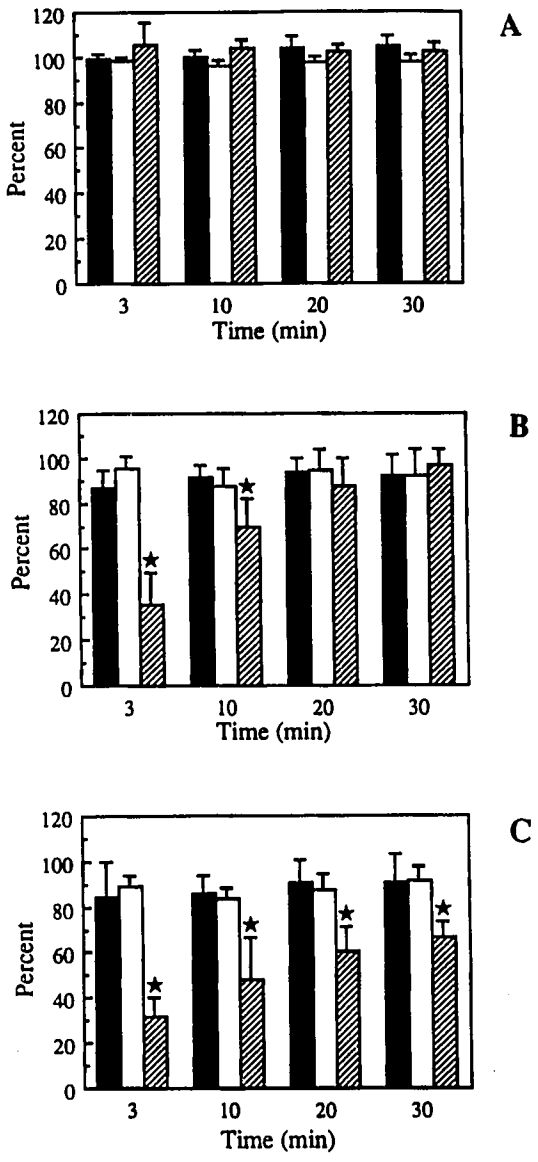

FIG. 5. The effect of the filter on (*A*) red blood cell counts, (*B*) white blood cell counts, and (*C*) palatelet counts. All data are plotted as percent of baseline before opening the fiber bundle. The data were presented as mean ± SD. Filled bars = group 1 animals (*i.e.*, the control dogs that received no protamine treatment); open bars = group 2 animals (*i.e.*, dogs with the protamine bio-reactor); and hatched bars = group 3 animals (*i.e.*, dogs that received intravenous administration of protamine). *$P < 0.05$ from the control group.

The white blood cell counts in animals receiving intravenous protamine administration decreased rapidly within the first 10 min after protamine administration, after which the counts began to rebound (fig. 5B). The transient decrease in white blood cell count was statistically significant ($P < 0.01$). When the protamine bio-reactor was used, there was only a slight decrease in the white blood cell counts. The white blood cell counts were almost unchanged at 3 min, decreased to 88 ± 8% of baseline at 10 min, returned to 95 ± 9% of baseline at 20 min, and remained constant thereafter. These latter changes were not statistically significant, when compared to those obtained from the control animals at the same time intervals.

Decreases in platelet counts appeared to follow a similar pattern (fig. 5C). In animals receiving protamine, the platelet counts declined to 32 ± 8% at 3 min, and then gradually rebounded to 67 ± 7% of baseline at 30 min. However, when the protamine bio-reactor was used, the maximum decrease in platelet count, which occurred at 10 min, was only to 84 ± 5% of baseline. None of these changes was statistically significant when the data were compared with those obtained from the control animals at the same time intervals.

No hemolysis appeared to occur during the operation of the protamine filter. The total hemoglobin remained constant at approximately 100% for both the control and the experimental animals. The normalized hematocrit also remained unchanged over the entire course of the experiment for all of the animals tested. The difference in the values of these hematologic parameters between the control group with the untreated sham reactor and the protamine bio-reactor group was negligible, suggesting that the protamine bio-reactor caused no further blood damage than was found with the untreated reactor.

The effect of the protamine bio-reactor on the complement system was examined. As shown in figure 6, the plasma complement level in the control animals, as measured by the CH_{50} hemolytic assay, gradually decreased over a period of 20 min until it reached a residual level of 80 ± 2% of baseline and remained constant thereafter. The changes were statistically significant ($P < 0.05$) when the data were compared to those at time zero. For animals receiving intravenous protamine treatment, however, the complement level was reduced much more rapidly. The residual complement level decreased to 80 ± 2% of baseline at 3 min and remained at the same level thereafter. These changes from the baseline were also statistically significant ($P < 0.05$). Conversely, for animals in which the protamine bio-reactor was used, the maximum decrease in plasma complement level, which occurred at about 20 min, was only to 90 ± 3% of baseline and was statistically significant ($P < 0.05$). In addition, this value was statistically different ($P < 0.05$) from those observed in the control group and in the group receiving intravenous protamine.

Discussion

In the *in vivo* experiments, the bio-reactors containing immobilized protamine removed heparin from the extracorporeal blood circuit. The two-compartment kinetic model for heparin removal seems ideally suited to the

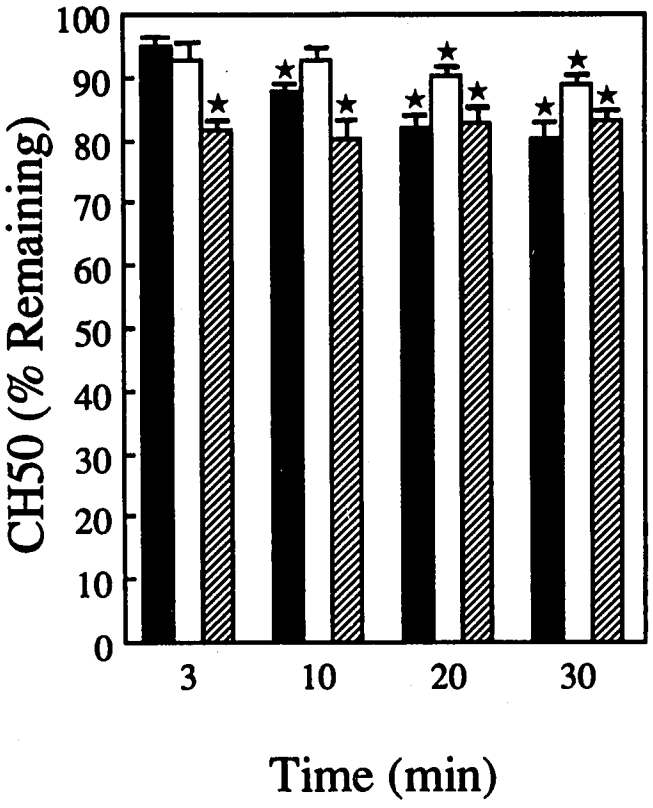

FIG. 6. The effect of the filter on the blood complement. Complement activation was measured by the total hemolytic assay (CH_{50} assay). All data are plotted as percent of baseline before opening the fiber bundle. The data are presented as mean ± SD. Filled bars = group 1 animals (*i.e.*, control dogs that received no protamine treatment); open bars = group 2 animals (*i.e.*, dogs attached with the protamine bio-reactor); and hatched bars = group 3 animals (*i.e.*, dogs that received intravenous administration of protamine). *$P < 0.05$ from the baseline.

accomplishment of both adequate extracorporeal heparinization as well as safe limitation of systemic anticoagulation. In some clinical settings, the heparin level cannot be increased sufficiently to prevent clotting of the devices because of the increased likelihood of severe hemorrhagic complications.[22] The use of the protamine bio-reactor in series with these extracorporeal devices may permit the safe use of adequate and higher doses of heparin in the circuit.

At an initial heparin concentration of 2 units/ml blood (calculated from the initial heparin dose of 150 units/kg body weight, assuming blood volume to make up 7% of total body weight of the animal), the rate of heparin removal by the protamine bio-reactor was relatively independent of the amount of protamine that was immobilized on the fiber bundle. Instead, the heparin removal rate was governed predominantly by the blood circulation rate, increasing at higher flow rates (data not shown). These results suggest that the protamine bio-reactor might be more effective in removing heparin if it is used in a cardiopulmonary bypass circuit with a flow rate of 3–4 l/min, rather than in hemofiltration or hemodialysis devices in which the flow rate is less than 500 ml/min.

When the protamine bio-reactor is considered for heparin removal in the clinical setting, several precautions must be kept in mind. One is that unlike intravenous protamine administration, which provides a systemic heparin reversal, the use of the protamine bio-reactor allows only a regional heparin removal. Thus, a full systemic removal of heparin anticoagulation by the protamine bio-reactor can never be achieved, because of the mixing of the filter-treated blood with the untreated blood arriving from other branches of the vascular system. As shown in figure 3, approximately 15% of the initial heparin activity still remained after the use of the protamine bio-reactor. The remaining heparin activity, however, might be effectively controlled to less than a certain low level (*e.g.*, < 0.2 unit/ml blood) consistent with a minimal risk of bleeding, by carefully selecting a protamine bio-reactor with the size and capacity adequate for each of the specific applications.

Another precaution is that coagulation of blood may occur in the circuit located between the protamine filter and the patient, where the heparin-depleted blood travels. A prophylactic regimen would be the use of a heparin coated material for this segment of the extracorporeal circuit. Heparin has been successfully linked to a variety of polymers,[23–26] and these heparin-coated materials have shown promising thromboresistant property.

A third precaution is that during the course of operation, the protamine bio-reactor gradually becomes saturated. This was shown by the abrupt decrease in heparin removal that occurred 10 min after the opening of the protamine bio-reactor (fig. 3). For the maintenance of a continuous, highly effective heparin removal, one alternative is the use of multiple reactors; while one reactor is in the process of removing heparin, the others could be regenerated by flushing the filters with high concentrations of salt solution. Further animal studies shall be directed to test this possibility.

The formation of clots on the fibers was found to be much more evident in animals receiving intravenous protamine than in those with the protamine bio-reactor. Several investigators have reported that heparin ionically bound to the surface of a biomaterial, including that of the protamine-coated material,[25] possesses antithrombogenic activity.[25,26] Shanberge *et al.*[27] suggested that in the presence of an excess of protamine, the large heparin–protamine complexes formed could activate antithrombin III. The thromboresistant property of the protamine bio-reactor may therefore be due to the antithrombogenicity of heparin that is ionically adsorbed to protamine.

Protamine-induced hypotensive responses were examined in dogs during the pilot phase of animal studies because the dog is one of the most economical animal

species for this type of acute experiments. In addition, the dog is known to magnify the typical human responses noted with protamine reversal of heparin.[28] Conahan and co-workers reported that the cardiovascular effects of protamine sulfate appeared to be more benign in humans than in the dogs.[29] Consistent with the findings by other investigators,[6,28] dogs with intravenous administration of protamine displayed important hemodynamic changes. Conversely, the use of the protamine bio-reactor did not elicit any statistically significant adverse hemodynamic response over the entire course of the experiment. Recent studies involving both animal species and human subjects suggest that complement activation and eicosanoid generation, particularly thromboxane, may be responsible for many of the acute manifestations observed during protamine reversal of heparin anticoagulation.[9,15,16,30,31] Both processes appear to depend on the amount of circulating protamine or heparin–protamine complexes.[15] As discussed previously, the use of a protamine bio-reactor would essentially prevent protamine and the heparin–protamine complexes from entering the circulation. Our results also indicate that the use of the protamine bio-reactor significantly reduced the activation of the complement system (fig. 6). The lack of hemodynamic response to the protamine filter may therefore be accounted for in terms of the absence of these two mechanisms.

The use of immobilized protamine significantly attenuated the protamine-induced thrombocytopenic response. Peripheral platelet counts were reduced by 68% in animals receiving intravenous protamine but only by 16% in animals with the protamine filter. Precisely how the immobilized protamine suppresses the thrombocytopenic response induced by free protamine remains unknown. Recent studies have suggested the presence of a correlation between thrombocytopenia and the development of hypotension.[13,28] The diminution in the degree of thrombocytopenia may therefore be linked to the lack of hypotensive response to immobilized protamine.

Numerous investigators have noted that transient granulocytopenia is manifested in patients undergoing dialysis[32] or protamine treatment.[16,33,34] This phenomenon has been linked to the reversible pulmonary leukosequestration resulting from the activation of granulocytes by complement-derived fragments.[16,32–34] Our results seem to agree with this mechanism. Animals that received intravenous protamine administration and demonstrated the highest degree of complement activation also were those that displayed the most significant decrease in white blood cell counts (*i.e.*, a decrease of 64%). On the other hand, animals in whom the protamine bio-reactor was used experienced the least degree of complement activation and showed a decrease of only 12% in white blood cell counts. Thus, the use of the protamine bio-reactor could also significantly diminish the granulocytopenic response associated with the use of protamine.

Like antibody-antigen complexes, Rent *et al.*[35] and Fiedel *et al.*[36] found that heparin–protamine complexes also activate the complement system. Consistent with their *in vitro* findings, our results showed that 3 min after protamine administration, the animal's plasma complement level had rapidly decreased by 20%. However, a reduction of only 10% of the CH_{50} values was observed in dogs treated with the protamine bio-reactor. A recent study conducted by Morel *et al.*[16] showed that a slower infusion rate of protamine for heparin reversal would significantly modify the responses to protamine. These results suggested that a reduction in the instantaneous circulating protamine concentration able to interact with free heparin molecules would yield a lesser degree of acute complement activation and anaphylatoxin generation. The induction of less complement activation by the protamine bio-reactor may therefore result from the binding of the heparin–protamine complexes to the reactor: since they are bound, they do not circulate in the blood stream and are thus unable to reach the target cells that in turn would release thromboxane. Fiedel *et al.*[36] reported that when heparin and protamine were combined in multiple proportions, the proportion that resulted in optimal precipitation also was the one that induced maximal complement consumption. It was therefore suggested that the heparin–protamine complex must achieve a critical size in order to activate C1q.[36,37] In the protamine bio-reactor, since protamine is covalently immobilized on the fiber surface, it presumably allows only adsorption of a single layer of heparin. Thus, the reduction in complement activation by the protamine bio-reactor may also be due to the lack of sufficient size for the heparin–protamine complex on the reactor to bind C1q.

It is noteworthy that the protamine bio-reactor induced even less complement activation (or consumption) than the untreated fiber bundle. The control dogs with the untreated fiber bundle displayed a decrease in the complement level that was twice as great as that observed in the animals with the protamine bio-reactor. Several reports have indicated that the presence of hydroxyl groups on the cellulose membrane would result in the activation of the complement system through the formation of thioester linkages with the complement components (C3 or C4).[38,39] In the protamine bio-reactor, since the hydroxyl groups on the cellulose fibers have been converted into isourea linkages (*i.e.*, the linkages between the protamine and the fiber), they may no longer be available for binding of the complement proteins. The decrease in complement activation by the protamine bio-reactor may also suggest that a protamine-coated cellulose membrane is more compatible with blood than is the original untreated membrane.

The most severe and persistent response to protamine appears to occur in patients who have been previously exposed to protamine.[7,28,40] This enhanced protamine

response is believed to be related to an immune-mediated mechanism.[29,40-43] In certain patients, immunoglobulin E or G reaginic antibodies are produced in response to protamine.[29,40-43] These antiprotamine antibodies are attached to mast cells present in the liver, lungs, and other organ tissues. A second exposure to protamine results in a reaction of protamine with its antibodies on the cell surface. Once the antigen–antibody complex is formed, it triggers the degranulation of the mast cell, releasing histamine and other vasoactive amines.[6,28,40] Although the use of immobilized protamine would not prevent protamine from interacting with antibodies that are fixed to circulating basophils, it would, however, block the conjugation of protamine to the antibodies that are fixed to the tissue mast cells. Since the basophils are present in low numbers relative to those of the tissue mast cells, the use of the protamine bio-reactor theoretically could significantly reduce the antibody-mediated anaphylactic response resulting from the second exposure to protamine. Research related to this possibility using protamine-sensitized animals will be conducted in our laboratory.

The current study suggests that the protamine bio-reactor under development may provide a unique means to control both heparin- and protamine-induced complications simultaneously. This control would significantly improve the safety of ECBC procedures. With nearly 20,000,000 such procedures performed each year, the benefits of the filter could be extensive. The improved blood compatibility and the reduced complement-activating ability of the protamine-bound hollow fibers may also enable them to replace those currently used in the construction of hemodialyzers.

From a practical standpoint, the protamine bio-reactor possesses two major advantages. One advantage is the simplicity and flexibility of operating the device. The bio-reactor can easily be interfaced with current ECBC devices without any additional apparatus or invasive procedures. The other advantage is the potential acceptance of the bio-reactor for clinical use. The bio-reactor is designed with a blood-compatible material (cellulose hollow fibers obtained from a clinical hemodialyzer) and a clinically accepted drug (protamine) and at the same time significantly minimizes the toxic effects of the drug. Its acceptance for clinical use, particularly in patients with increased risk of bleeding complications from required heparin use, would therefore be anticipated.

More generally, the concept discussed in this paper may open up the possibility for the use of other immobilized chemicals for extracorporeal blood treatment, particularly when these chemicals have potential toxic side effects and cannot be administered safely into the vascular system.

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