CARDIOPULMONARY BYPASS, MYOCARDIAL MANAGEMENT, AND SUPPORT TECHNIQUES

NITRIC OXIDE INHIBITION ATTENUATES SYSTEMIC HYPOTENSION PRODUCED BY PROTAMINE

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Background: **Protamine reversal of heparin anticoagulation often causes systemic hypotension, and in vitro studies suggest that this may be mediated by release of nitric oxide from the endothelium. The present investigations were designed to evaluate the direct myocardial effects of protamine and to determine in vivo whether nitric oxide inhibition can prevent hypotension during protamine infusion.** *Methods/Results:* **Protamine sulfate (50 μg/ml) was added to perfusate of eight isolated rabbit heart preparations; in six other preparations, a similar concentration of protamine was added to *heparinized* (5 U/ml) Krebs perfusate. Left ventricular developed pressure, maximum rate of pressure rise, and heart rate declined significantly ($p < 0.01$) in hearts exposed to protamine only (65.0% ± 6.6%, 55.5% ± 6.0%, and 87.6% ± 2.5% of baseline, respectively), whereas protamine added to heparinized perfusate caused little change in developed pressure, maximum rate of pressure rise, and heart rate (85.3% ± 5.4%, 84.9% ± 5.5%, and 98.8% ± 1.6%). To study systemic effects of protamine, we measured hemodynamic parameters in 12 heparinized dogs (150 U/kg). During protamine infusion (1.5 mg/kg intravenously over 30 seconds), mean blood pressure decreased by 46% ± 7% from baseline ($p < 0.05$), cardiac output decreased by 38% ± 4% ($p < 0.05$), and systemic vascular resistance decreased by 14% ± 9%. After hemodynamic stabilization, N^g-monomethyl-L-arginine (2 mg/kg), a competitive inhibitor of nitric oxide synthesis, was administered to six dogs, and methylene blue (2 mg/kg), an inhibitor of cyclic guanosine monophosphate synthesis, was administered to the remaining six dogs. After treatment with N^g-monomethyl-L-arginine and methylene blue, the second infusion of protamine sulfate caused no significant change in blood pressure or cardiac output. In an additional six dogs, N^g-monomethyl-L-arginine pretreatment (5 mg/kg) blocked the effects of the first dose of protamine. The effect of N^g-monomethyl-L-arginine could be reversed by the addition of (6 mg/kg) L-arginine but not D-arginine.** *Conclusions:* **Protamine-heparin complex does not cause direct myocardial depression but does lead to severe hypotension in vivo. The finding that hypotension can be blocked by inhibitors of the nitric oxide pathway confirms previous in vitro studies indicating that the effects of protamine are mediated, in part, by the vascular endothelium. Further, these studies suggest a novel approach to prevention of hemodynamic complications caused by heparin reversal after cardiopulmonary bypass. (J Thorac Cardiovasc Surg 1996;111:1240-7)**

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Read at the Twenty-first Annual Meeting of The Western Thoracic Surgical Association, Coeur d'Alene, Idaho, June 21-24, 1995.

Received for publication June 21, 1995; revisions requested Oct. 4, 1995; revisions received Oct. 9, 1995; accepted for publication Jan. 18, 1996.

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0022-5223/96 $5.00 + 0 **12/6/72120**

Protamine sulfate is a polycationic protein used to reverse the anticoagulant effect of heparin in cardiovascular operations.[1] The action of protamine is thought to result from the one-to-one cationic-anionic pairing with heparin to achieve reversal.[2-5] However, the use of protamine has been associated with numerous clinically significant side effects, including systemic hypotension, pulmonary vasoconstriction, and anaphylaxis.[6-8] The most common untoward effect is systemic hypotension, and reports of hypotensive reactions to protamine have become more frequent with the widespread use of cardiac catheterization and cardiopulmonary bypass.[9-13] Such adverse reactions can be especially dangerous in the period immediately after extracorporeal circulation if cardiac function is impaired. Thus prevention of hypotension during protamine administration is especially important to cardiac surgeons and anesthesiologists.[14-18]

Recent evidence suggests that protamine has a direct effect on myocytes. Hird and colleagues[19] demonstrated that in isolated myocyte preparations, unbound protamine directly depresses myocyte contractility and blunts its β-adrenergic responsiveness; importantly, the heparin-protamine complex did not have this effect. Furthermore, studies from our laboratory by Pearson and associates[20] established a possible mechanism for vasodilation in response to protamine; in vitro, protamine stimulates release of nitric oxide from the vascular endothelium.

In light of these recent findings, we designed the present experiments to evaluate further the direct myocardial and systemic effects of protamine and to examine whether the vascular endothelial release of nitric oxide plays a role in the development of hypotension in vivo.

Materials and methods

To study the central and systemic effects of protamine sulfate infusion, we performed two sets of experiments. First, in vitro isolated rabbit hearts were used to evaluate the effect of protamine on myocardial function. Second, an intact canine preparation was used to determine the systemic hemodynamic effects of protamine. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH Publications No. 85-23, revised 1985), and protocols were approved by the Institutional Animal Care and Use Committee of the Mayo Foundation.

Modified Langendorff preparation (in vitro studies). Adult New Zealand White rabbits (either sex, 3.0 to 4.0 kg) were anesthetized by intravenous injection of pentobarbital (35 mg/kg body weight), and cardiectomy was performed. Beating hearts were immediately placed in a cold (4° C) modified Krebs-Ringer-bicarbonate (KRB) solution of the following millimolar composition: NaCl, 118.3; KCl, 4.7; $MgSO_4$,1.2; KH_2PO_4, 1.22; $CaCl_2$, 2.5; $NaHCO_3$, 25.0; and glucose, 11.1. Hearts were then suspended from a Langendorff perfusion column, and retrograde perfusion was established at 37° C with an overflow system to maintain a constant coronary perfusion pressure of 70 mm Hg. The time delay between harvest and perfusion of all hearts was kept to less than 2 minutes. The nonrecirculating KRB perfusate was bubbled with a 95% oxygen and 5% carbon dioxide gas mixture to achieve a perfusate oxygen tension more than 600 mm Hg and pH 7.4, and the solution was filtered. After retrograde perfusion was established, the free wall of the left atrium, the mitral valve leaflets, and the chordae tendineae were excised to vent the left ventricle and permit insertion of an oversized intraventricular balloon; this was attached to a flange, which was secured to the mitral valve anulus. A water-filled tube (PE190; Clay-Adams, Becton-Dickinson Co., Parsippany, N.J.) connected the balloon to a transducer (Gould 2400, Gould, Inc., Cleveland, Ohio) for measurement of left ventricular developed pressure, the maximal rate of rise of left ventricular pressure (dp/dt), and heart rate. Timed collections of coronary effluent were made for measurement of coronary flow rate.

Protocol. Hearts were randomly divided into two groups. In the protamine group (n = 8), hearts were stabilized for 20 to 30 minutes and then exposed to protamine at a final concentration of 50 μg/ml (protamine sulfate, Lyphomed, Inc., Rosemont, Ill.) for 20 minutes (experimental period). In the heparin-protamine group (n = 6), the hearts were exposed to protamine in the same manner as the first group; however, the KRB solution contained 5 U/ml concentration of heparin (heparin sodium, Elkins-sinn, Inc., Cherry Hill, N.J.) throughout the experiment. A 50 μg/ml dose of protamine is approximately equal to 4 mg/kg of body weight and can reverse a heparin concentration of 5 U/ml, which is approximately equal to 400 U/kg of body weight. This concentration of heparin is similar to that used during cardiopulmonary bypass.

Left ventricular developed pressure, dp/dt, heart rate, and coronary flow rate were measured just before (baseline) and after exposure to protamine (at 3, 5, 10, 15, and 20 minutes). Addition of heparin or protamine, or both, did not alter the other constituents of the KRB solution.

Data analysis. The results were expressed as percent change from control, and data are summarized as mean ± standard error of the mean. Statistical evaluation was performed by means of two-way analysis of variance for comparing time-related changes between groups. Then, pairwise comparisons were made by means of the Newman-Keuls multiple comparisons test. Differences were considered to be statistically significant when the p value was less than 0.05.

Canine experiments (in vivo studies). In vivo experiments were performed in 20 heartworm-free mongrel dogs (25 to 32 kg) anesthetized with pentobarbital sodium (30 mg/kg total dose). All dogs underwent median sternotomy and were monitored with a femoral arterial catheter to measure blood pressure. A Swan-Ganz pulmo-

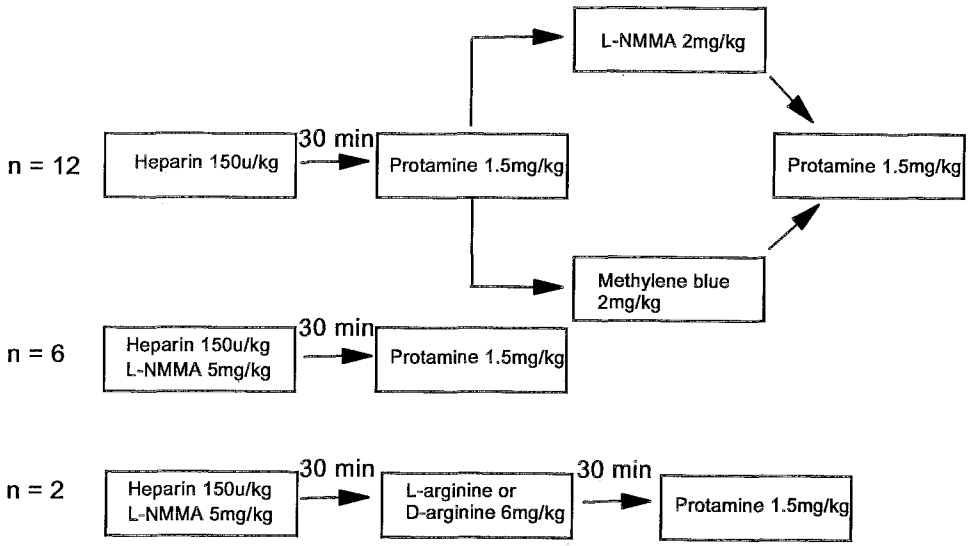

Fig. 1. Protocol for in vivo experiments. *n,* Number of animals studied.

nary artery catheter (Baxter Healthcare Corp., Edwards Div., Santa Ana, Calif.) was used to monitor intracardiac pressures and to measure cardiac output by the thermodilution method. Hydration in the animals was maintained by constant intravenous infusion of Ringer's lactate solution (10 ml/min) throughout the experiment. Standard hemodynamic measurements including heart rate, systemic and pulmonary arterial blood pressures, right atrial pressure, and cardiac output were made before and after each intervention.

Protocol. The study protocol is shown in Fig. 1. The first group of 12 dogs was given an intravenous heparin dose of 150 U/kg (heparin sodium, Elkins-sinn, Inc.), and 30 minutes later protamine sulfate, 1.5 mg/kg (protamine sulfate, Lyphomed, Inc.), was administered intravenously over 30 seconds. These doses of heparin and protamine corresponded to one-to-one reversal. After stabilization from the first dose of protamine, N^g-monomethyl-L-arginine 10^{-3} (L-NMMA) (2 mg/kg) (Calbiochem, San Diego, Calif.), a competitive inhibitor of nitric oxide synthesis from L-arginine, was administered as a bolus infusion in six dogs, and methylene blue (2 mg/kg) (Sigma Chemical Company, St. Louis, Mo.), an inhibitor of cyclic guanosine monophosphate, was administered in the other six dogs.

To control for possible confounding effects of repeated protamine administration, we studied an additional six dogs. Animals were heparinized (150 U/kg) and then pretreated with L-NMMA before protamine reversal of heparin. In two additional dogs, L-arginine and D-arginine were administered to overcome the blockade of L-NMMA.

Data analysis. Results were expressed as percent change from the control (preintervention) value and are summarized as means ± standard error of mean. Data were analyzed by means of two-way analysis of variance and Student's *t* test when appropriate. Differences were considered to be statistically significant when p was less than 0.05.

Results

In vitro studies. During the 20-minute study period, left ventricular developed pressure, maximum dp/dt, and heart rate declined significantly in a time-dependent manner in the isolated hearts exposed to protamine only (65.0% ± 6.6%, 55.5% ± 6.0%, and 87.6% ± 2.5% of baseline at 20 minutes, respectively). As seen in Fig. 2, these adverse effects of protamine were attenuated by heparin. In hearts perfused with *heparinized* KRB, protamine caused little change in left ventricular developed pressure, maximum dp/dt, and heart rate (85.3% ± 5.4%, 84.9% ± 5.5%, and 98.8% ± 1.6% of baseline, respectively), and the differences between groups with and without heparin were statistically significant ($p < 0.01$ by two-way analysis of variance). In contrast to other variables, coronary flow was well preserved (91.4% ± 8.1%) in the protamine group and in the protamine-heparin group (84.7% ± 5.6%).

In vivo studies. Without pretreatment, protamine reversal of heparin caused a significant decrease in mean blood pressure (decrease of 46% ± 7% from baseline, $p < 0.05$), cardiac output (decrease of 38% ± 4% from baseline, $p < 0.05$), and systemic vascular resistance (decrease of 14% ± 9% of baseline). In these dogs, L-NMMA and methylene blue markedly attenuated the hemodynamic disturbance after the second dose of protamine (Fig. 3).

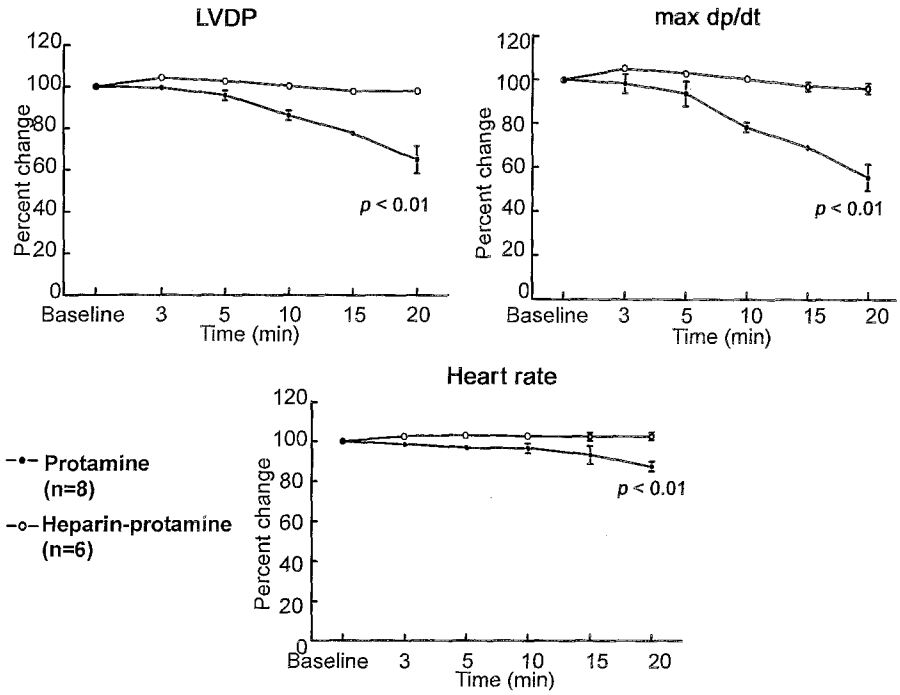

Fig. 2. Effect of protamine-heparin complex on left ventricular developed pressure *(LVDP)*, maximum dp/dt, and heart rate. The protamine group showed a significant decline during the 20-minute experimental period ($p < 0.01$, one-way analysis of variance). The presence of heparin attenuated protamine's effect. There is a statistically significant difference between the two groups ($p < 0.01$, two-way analysis of variance).

In the dogs pretreated with L-NMMA, the initial dose of protamine had little effect on systemic hemodynamics (mean arterial pressure decreased 3% ± 6%, cardiac output decreased 5% ± 5%, and systemic vascular resistance rose 4% ± 8% from baseline) (Fig. 4).

An additional two dogs received pretreatment with L-NMMA; administration of L-arginine overcame the effect of the competitive blocker, and protamine caused hypotension. However, blood pressure remained stable when D-arginine was given to overcome competitive inhibition of nitric oxide synthase (Fig. 5).

Discussion

The administration of protamine to reverse heparin may lead to systemic hypotension, and this can be a catastrophic event early after cardiopulmonary bypass, especially in patients with impaired ventricular function. Numerous studies have investigated possible mechanism(s) for these adverse reactions. In vitro studies by Wakefield and colleagues[21, 22]

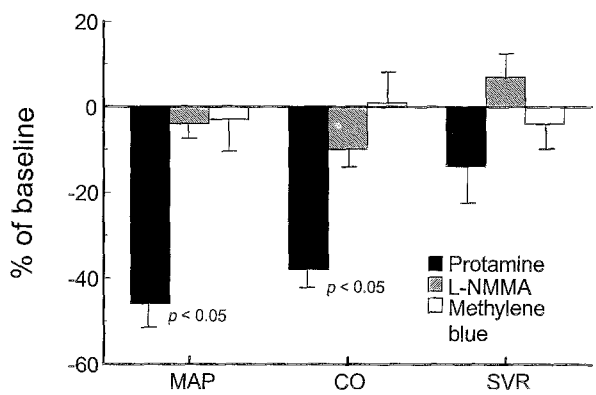

Fig. 3. Results from the initial response to protamine are designated as control and are compared with the hemodynamic measurements to repeated protamine infusion after treatment with L-NMMA ($n = 6$) or methylene blue ($n = 6$). Mean arterial pressure *(MAP)* and cardiac output *(CO)* are maintained as compared with control animals receiving only protamine ($n = 12$) ($p < 0.05$, Student's t test). Although the systemic vascular resistance *(SVR)* is not decreased as in hearts receiving only protamine, the difference is not statistically significant.

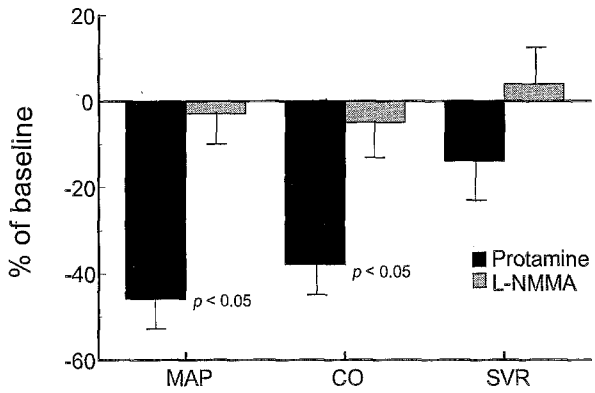

Fig. 4. Results from the initial response to protamine are shown as control. These are compared with the hemodynamic measurements seen after pretreatment of the dogs with L-NMMA (5 mg/kg) ($n = 6$). Mean arterial pressure *(MAP)* and cardiac output *(CO)* are well preserved in the L-NMMA group, and this difference is statistically significant ($p < 0.05$ Student's t test). Systemic vascular resistance *(SVR)* does not decrease as in the control group, but this difference is not statistically significant.

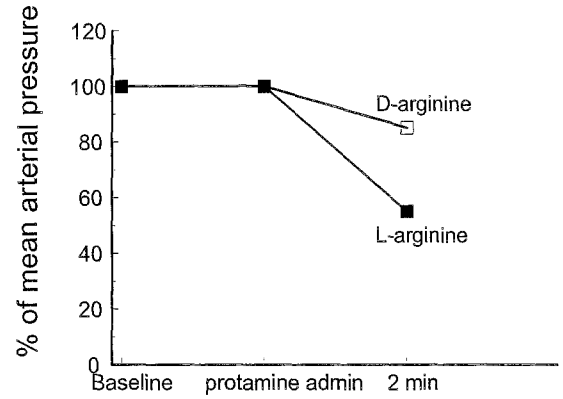

Fig. 5. Results from two dogs are shown. Percent change of mean arterial pressure from baseline is compared in an animal receiving L-arginine (6 mg/kg) to an animal receiving D-arginine (6 mg/kg) to overcome the blockade by L-NMMA pretreatment. The animal receiving L-arginine has profound systemic hypotension (decrease of 55% from baseline) on protamine infusion. The response to protamine is less in the animal receiving D-arginine (decrease of 15% from baseline).

suggested that protamine alone could cause dose- and time-dependent decrements in myocardial performance in the isolated rabbit heart. However, their investigation did not examine the effects of the heparin-protamine complex on myocardial function. More recently, Hird and associates[19] demonstrated in isolated myocyte preparations that unbound protamine directly depresses myocyte contractility and blunts its β-adrenergic responsiveness, but the heparin-protamine complex did not have this effect.

To assess the myocardial effects of the heparin-protamine complex, we used an isovolumic rabbit heart model. The concentration of heparin and protamine in the protocol closely paralleled the clinical use of anticoagulation and allowed us to examine the effects of the protamine and heparin in a beating heart.

In these experiments, measures of left ventricular performance (developed pressure and maximum dp/dt) were significantly reduced when the rabbit hearts were exposed to protamine alone. These parameters changed very little when protamine was added to heparinized perfusate. Importantly, the model provides isovolumic measures of ventricular performance and a constant perfusion pressure; these features are critical in evaluation of compounds that have effects on systemic vascular resistance. It appears, therefore, that the protamine-heparin complex has little direct myocardial effect.

These results are also consistent with the clinical observations of less hemodynamic disturbance with slow administration of protamine versus rapid infusion and less hemodynamic disturbance with intraaortic administration compared with intravenous infusion.[16, 18] It is possible that with rapid infusion, especially rapid intravenous infusion, uncomplexed protamine is presented to the coronary circulation ("first pass" effect), and the resulting direct myocardial depression may aggravate hypotension caused by reduction in systemic vascular resistance. Slow infusion of protamine and intraaortic administration minimize the chance that unbound protamine will enter the coronary circulation.

Clinical studies by Shapira and coworkers[6] reported that the major hemodynamic consequence of protamine administration early after cardiopulmonary bypass was peripheral vasodilatation. In that study, 10 patients (45%) had mild peripheral vasodilatation with an increase in cardiac output sufficient to prevent a reduction of blood pressure. In contrast, peripheral vasodilatation was more profound in the remaining 12 patients (55%) and was associated with impairment of myocardial contractility, as evidenced by a decrease in contractile element velocity. Thus all patients studied had some change in peripheral vascular resistance. This finding, along with the in vitro studies by Pearson and colleagues,[20] which showed that protamine can

cause arterial vasorelaxation by endothelial release of nitric oxide, suggest that the effect of protamine in human beings might, in a large part, be due to peripheral vasodilatation.

In our canine model, administration of a 1.5 mg/kg dose of protamine consistently led to profound hypotension (mean decrease of 46% from baseline). When pretreated with a specific, competitive inhibitor of the nitric oxide pathway, N-NMMA, hemodynamic parameters after protamine infusion remained stable. The competitive inhibition of nitric oxide synthase was overcome by the addition of L-arginine and not D-arginine. These are the first in vivo studies to implicate nitric oxide release with protamine-induced hypotension.

It is tempting to ascribe the systemic hypotension in experiments to a *direct* action of protamine on the peripheral vasculature mediated by nitric oxide release. However, the precise mechanism may be more complicated. In prior in vitro studies,[20] the time course of the effect of protamine on systemic vessels was gradual, and complete relaxation to a bolus infusion of protamine required at least 10 minutes. Systemic hypotension observed in these in vivo experiments (and that seen in human beings) occurs much more quickly, usually within 2 minutes after protamine infusion. Perhaps nitric oxide release is the final mediator of hypotension during protamine infusion in clinical practice, and the triggering events are initiated by other humoral pathways.

Also unexplained was the finding that in the canine experiments we noted a greater decrease in cardiac output than would be expected with changes in peripheral resistance alone. In the rabbit heart preparation, protamine, but not the protamine-heparin complex, led to a decrease in myocardial performance independent of preload and afterload variations. It is possible that a relatively small decrease in arterial and venous tone decreases ventricular filling, which in turn reduces cardiac output and worsens hypotension. Also, there may be a secondary myocardial effect of protamine resulting from myocardial ischemia caused by a fall in coronary perfusion pressure. Finally, it is possible that there is direct myocardial depression in our canine experiments that is species specific and thus different from the rabbit model.

Another explanation for the marked reduction in cardiac output is pulmonary vasoconstriction. Certainly pulmonary vasoconstriction might reduce filling of the left side of the heart and thereby reduce cardiac output. This investigation was not designed to precisely quantify changes in pulmonary hemodynamics and in vivo cardiac function during protamine infusion. Current studies in our laboratory do address these important effects of protamine.

Other mechanisms that might be involved in protamine-induced hypotension were not evaluated in our protocol. The arachidonic acid pathway and its metabolites have been implicated as mediators of protamine-induced pulmonary vasoconstriction by Wakefield and colleagues.[23] In human beings, protamine clearly causes complement activation.[7] By-products or end products of these pathways (or both) may play a role in protamine-induced hypotension, and further investigation into their relationship to nitric oxide release is warranted.

Summary

This investigation suggests that uncomplexed protamine has a significant negative inotropic effect, but the protamine-heparin complex causes minimal myocardial depression of the intact myocardium. Furthermore, systemic hypotension might occur as a result of endothelial release of nitric oxide in vivo, and this can be attenuated by inhibition of the nitric oxide pathway. This is the first study to suggest that the nitric oxide pathway might be an important mediator of adverse reactions attributed to protamine in vivo and offers a novel approach to prevention of hypotension caused by heparin reversal after cardiopulmonary bypass.

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Discussion

Dr. Walter P. Dembitsky *(San Diego, Calif.)*. The experiments presented are somewhat complicated, making statistical analysis also somewhat complicated. Nonetheless, according to our statistician, the conclusions should remain the same. In the series of in vitro studies in which a modified Langendorff preparation was used to analyze the function of rabbit hearts, it seems clear that protamine does directly suppress myocardial function. The suppression is not present when protamine is combined with heparin. It is intriguing that the coronary blood flow remains unchanged despite reductions in major determinants of myocardial oxygen consumption, heart rate, developed left ventricular pressure, and peak dp/dt. This suggests that the coronary autoregulation is disturbed. Does protamine dilate coronary arteries as well?

Dr. Raikar. In vitro studies by Pearson and colleagues demonstrate that protamine alone can cause coronary vasorelaxation. In vivo, there might be a chance for uncomplexed protamine to be seen by the coronary circulation. The protamine-heparin complex, however, does not seem to have direct effects on the coronary circulation.

Dr. Dembitsky. The clinical phenomenon of transient reduction of blood pressure after heparin administration is common and is undoubtedly more significant in patients with compromised cardiac function. It does appear from your work that this hypotension can be induced by the direct or indirect influences of protamine on the peripheral vascular release of nitric oxide. You suggest there may be novel approaches that interfere with nitric oxide release and prevent protamine-induced hypotension. I wonder if reduction in systemic vascular resistance in human beings is the most important determinant of systemic hypotension. The time required in vitro for protamine to gradually and maximally relax the systemic vessels is about 10 minutes. This is often longer than experienced clinically and longer than you saw in your in vivo experiments. The fall in cardiac output you observe is greater than you expected with the changes in systemic vascular resistance. In your canine preparation, you did measure the pulmonary artery pressure and cardiac output and could have measured pulmonary capillary wedge pressure. The pulmonary data were not included in the manuscript. Did you determine the changes in pulmonary vascular resistance in your experiments?

Dr. Raikar. We measured changes in pulmonary vascular resistance primarily through changes in right atrial pressure in the open chest dog. There was a tendency toward a decrease in filling pressures in these animals. Current studies in our laboratory have implemented more sensitive measures of cardiac function (Emax) and of the pulmonary vasculature.

Dr. Dembitsky. In our clinical experience, the peripheral vasodilatation and myocardial suppressive effects of protamine seem to be more easily managed than the occasional episodes of severe pulmonary vasoconstriction, which can produce severe systemic hypotension. Although this can often be an allergic phenomenon, pulmonary vasoconstriction may also be mediated

through other humoral mechanisms that can simultaneously produce systemic hypotension. Vasoactive mediators may exert their influence before the delayed protamine–nitric oxide interaction you have documented. Do you consider the relation of protamine administration and systemic hypotension in dogs to be a direct or indirect effect?

Dr. Raikar. From the present studies we can conclude that there is probably an indirect mechanism at work. Certainly other studies have shown that complement activation and the arachidonic acid pathway are also mediators involved in these reactions, and perhaps nitric oxide is the final mediator of these adverse reactions.

Dr. Dembitsky. Do you think interfering with endothelial nitric oxide release can cause pulmonary hypertension?

Dr. Raikar. Perhaps in higher doses of L-NMMA this might occur. In vivo studies of endothelium-induced sepsis have used very high doses (20 to 30 mg/kg) of L-NMMA. Our studies used doses of 2 to 5 mg/kg, and at these doses we have not observed any pulmonary hypertension.

Dr. Dembitsky. Last, what do you think the relevance of your canine findings are in human beings?

Dr. Raikar. I think it is an important first step to evaluate the clinical use of L-NMMA and other blockers of the nitric oxide pathway. We are in the process of concluding another study in the canine model, and studies in patients undergoing cardiopulmonary bypass will enlighten us as to the efficacy of these new modalities.