The effects of protamine sulfate on clot formation time and clot strength thromboelastography variables for canine blood samples

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Objective—To determine the effects of protamine sulfate on clot formation time and clot strength thromboelastography variables for canine whole blood samples.

Animals—Blood samples obtained from 11 healthy dogs.

Procedures—Blood samples were collected from jugular veins of dogs into syringes with 3.2% sodium citrate (blood to citrate ratio, 9:1). Blood samples were divided into aliquots, and protamine sulfate was added to various concentrations (0 [control], 22, 44, and 66 µg/mL). Prepared samples were activated with kaolin (n = 8) or not activated (8), CaCl₂ was added, and thromboelastography was performed. Reaction time (R), clot formation time (K), rate of clot formation (α angle), and maximum amplitude (MA) were measured.

Results—For kaolin-activated and nonactivated blood samples, protamine (66 µg/mL) significantly increased R and K and decreased α angle and MA, compared with values for control samples. Also, protamine (44 µg/mL) decreased MA in nonactivated blood samples and increased K and decreased α angle in kaolin-activated samples, compared with values for control samples.

Conclusions and Clinical Relevance—Results indicated protamine prolonged clot formation time and decreased overall clot strength in a dose-dependent manner; such effects may contribute to a hypocoagulable state in dogs. Kaolin-activated and nonactivated blood samples were appropriate for measurement of the effects of protamine on coagulation. Administration of protamine to reverse the effects of heparin should be performed with caution. (Am J Vet Res 2014;75:338–343)
Thromboelastography allows for the detection of hypercoagulable or hypocoagulable states, clotting factor deficiencies, and thrombocytopenias and has been used to guide selection of blood products.\textsuperscript{21-23} The use of thromboelastography reduces the total number of blood products administered to human patients.\textsuperscript{24-26} Both native (nonactivated) and kaolin-activated blood samples obtained from healthy dogs can be analyzed with that method.\textsuperscript{30,31} but some authors\textsuperscript{30-32} have suggested that the use of coagulation activators may mask changes in coagulation variables for patients with coagulopathies. The effects of protamine on thromboelastography variables for dogs have not been determined, to the authors’ knowledge.

The purpose of the study reported here was to determine the effects of protamine on thromboelastography variables for clot formation time and clot strength in whole blood samples obtained from dogs. We hypothesized that protamine would increase the time to clot formation and decrease clot strength. We also hypothesized that the use of kaolin for activation of coagulation during thromboelastography would decrease the effects of protamine.

**Materials and Methods**

**Samples**—This study was approved by the Institutional Animal Care and Use Committee of Michigan State University, and written informed consent was obtained from each dog owner. For each of the 2 thromboelastography experiments in this study (assay performed with and without kaolin activation), blood samples were collected from 8 privately owned dogs that were determined to be healthy on the basis of histories and results of physical examinations. Blood samples obtained from 5 dogs were used in both experiments, and blood samples from 6 dogs were used in 1 experiment; therefore, blood samples were collected from a total of 11 dogs for this study. The dogs included 8 castrated males and 3 spayed females. Breeds included 8 mixed-breed dogs, 2 German Shepherd Dogs, and 1 Border Collie. Median age of the dogs was 4 years (range, 3 to 8 years). For the 5 dogs with blood samples that were used in both experiments, at least 24 hours was allowed between collections of blood samples. All animals were fed a maintenance food intended for dogs. Dogs were excluded from the study if they were receiving any medications other than prophylactic parasiticides.

Prior to performance of experiments, thromboelastography variables were determined for each dog; values were within reference intervals for the laboratory. In addition, serum total solids concentration, PCV, and platelet counts were determined for each blood sample. Platelet counts were determined with an established protocol\textsuperscript{33} by a single investigator (CJB). Briefly, blood smears were prepared with citrated whole blood samples after samples were kept at room temperature for 30 minutes. Slides were examined by means of light microscopy to detect large platelet clumps, and the number of platelets in 10 hpf was counted and multiplied by 15,000; the mean value for the 10 hpf was determined. Blood samples were not used if substantial platelet clumping was detected or platelet counts were outside the laboratory reference range for dogs.\textsuperscript{33} Serum total solids concentrations were measured by use of a refractometer. Packed cell volume was measured after centrifugation of heparinized capillary tubes\textsuperscript{a} at 13,000 \times g for 3 minutes. Blood samples were determined to have clinically normal platelet counts (median, 146,000 platelets/µL; range, 96,000 to 220,000 platelets/µL), serum total solids concentrations (median, 5.9 g/dL; range, 5.7 to 5.9 g/dL), and PCVs (median, 44%; range, 38% to 50%).

**Study protocol**—Food was withheld from dogs overnight before collection of blood samples. A blood sample (5.4 mL) was collected from a jugular vein of each dog with a 20-gauge needle into a 6-mL syringe containing 0.6 mL of 3.2% sodium citrate (total sample volume, 6 mL). Each blood sample was collected by means of direct needle entry into the vein, and all samples used in the study were collected on the first attempt (if blood samples were not collected on the first attempt, no further attempt was made to collect blood samples from the dog on that day). Blood samples were kept at room temperature (22.5°C) for 30 minutes prior to preparation for thromboelastography. Blood samples were divided into aliquots for 4 experimental groups of protamine sulfate\textsuperscript{a} concentrations: 0 µg of protamine/mL (control), 22 µg of protamine/mL (intended to simulate the circulating concentration in a dog that received a dose of 2 mg/kg), 44 µg of protamine/mL (intended to simulate a dose of 4 mg/kg), and 66 µg of protamine/mL (intended to simulate a dose of 6 mg/kg). A theoretical total blood volume of 90 mL/kg in each animal was used for calculation of the approximate circulating concentration of protamine that would be achieved in a dog after administration of a dose. For preparation of blood samples with protamine, protamine (10 mg/mL) was diluted with saline (0.9% NaCl) solution\textsuperscript{a} so that the total volume of each sample was equal, regardless of the protamine concentration. For the 22 µg/mL preparation, 7.5 µL of protamine was mixed with 92.5 µL saline solution. For the 44 µg/mL preparation, 15 µL of protamine was mixed with 85 µL of saline solution. For the 66 µg/mL preparation, 22.5 µL of protamine was mixed with 77.5 µL of saline solution. The final ratio of blood to protamine solution for each aliquot was 33:1.

For performance of thromboelastography without kaolin activation, blood samples were mixed by means of repeated inversion and 990 µL of blood was mixed with 30 µL of the protamine and saline solution mixture prepared for each protamine concentration group. Each mixture was inverted 5 times to ensure proper mixing, and thromboelastography\textsuperscript{a} was performed.

For performance of thromboelastography with kaolin activation, blood samples were mixed by means of repeated inversion and 1,188 µL of blood was mixed with 36 µL of the protamine and saline solution mixture prepared for each protamine concentration group. Each mixture was inverted 5 times to ensure proper mixing, a 1,000-µL aliquot was pipetted into a tube containing kaolin,\textsuperscript{a} samples were mixed by means of repeated inversion, and thromboelastography was performed.

**Thromboelastography**—A 340-µL aliquot of each prepared blood sample was added to a warm (39°C)
thromboelastography cup, and 20 µL of 0.2M CaCl₂ was added. Thromboelastography was performed at 39°C (intended to simulate the approximate rectal temperature of dogs). The R (reaction time), K (clot formation time), α angle (rate of clot formation), and MA (a measure of overall clot strength) were measured for each sample. Each blood sample was assayed in 1 thromboelastography well; duplicate samples were not assayed because only 4 thromboelastography wells were available. The well that was used for each assay was determined by means of a randomization procedure (random number generator). Assay-specific quality control samples were assayed in all 4 thromboelastography wells on each day prior to data collection for experimental samples.

Statistical analysis—Statistical analysis was performed with a commercially available statistical software program. For each assay condition (nonactivated and kaolin-activated thromboelastography), R, K, α angle, and MA values were compared separately. Data were analyzed with a Friedman's test. For very high values that were considered essentially infinite (ie, clots did not form), data were assigned the highest rank. Values of P < 0.05 were considered significant.

Results

Thromboelastography without kaolin activation—The median R and K values were significantly longer for blood samples with 66 µg of protamine/mL (R, 25.6 minutes; K, 28.4 minutes) than they were for samples with 0 µg of protamine/mL (R, 10.9 minutes; K, 8.4 minutes). The median α angle was significantly smaller for blood samples with 66 µg of protamine/mL (8.1° and 26.6°, respectively). The median MA was significantly smaller for blood samples with 66 µg of protamine/mL (32.2 and 26.6 mm, respectively) versus samples with 0 µg of protamine/mL (50.9 mm). No significant differences in values were detected between
blood samples with 22 µg or protamine/mL and those with 0 µg of protamine/mL or among samples with 22, 44, or 66 µg of protamine/mL.

**Thromboelastography with kaolin activation—**
The median R value was significantly longer for blood samples with 66 µg of protamine/mL (17.1 minutes) than it was for samples with 0 µg of protamine/mL (3.9 minutes). The median K value was significantly longer for blood samples with 44 or 66 µg of protamine/mL (12.6 and 12.2 minutes, respectively) than it was for samples with 0 µg of protamine/mL (3.9 minutes). The median α angle was significantly smaller for blood samples with 44 or 66 µg of protamine/mL (16.2° and 17.9°, respectively) than it was for samples with 0 µg of protamine/mL (44.3°). Blood samples with 66 µg of protamine/mL had a significantly smaller median MA versus those with 0 µg or protamine/mL (37.7 and 45.9 mm, respectively). No significant differences in values of variables were detected between blood samples with 22 µg of protamine/mL and those with 0 µg of protamine/mL or among samples with 22, 44, or 66 µg of protamine/mL.

**Discussion**
Performance of thromboelastography with whole blood samples allows global assessment of coagulation. Four variables are typically evaluated for thromboelastography results. The R and K times represent the time required for a clot to start to develop, the α angle represents the rapidity of clot formation, and the MA represents overall clot strength. Results of the present study indicated protamine increased R and K times and decreased α angle and MA of blood samples. Therefore, as assessed by means of thromboelastography, protamine delayed clot formation and decreased clot strength and these effects seemed to be dose dependent. Although protamine is known to have anticoagulant properties, the present study is the first in which the effects of protamine on thromboelastography variables for canine whole blood samples were determined, to the authors’ knowledge. A similar study was conducted with human plasma samples; results indicate protamine significantly delays clot formation and decreases clot strength. However, because that study was conducted with plasma samples, the effects of protamine on platelets and other blood cells during clot formation were not de-
The pharmacokinetics of protamine is poorly understood. However, protamine likely induces a hypo-
coagulable state by means of inhibition of coagulation factor V and modulation of platelet activity. Activation of coagulation factor V to factor Va enhances the ability of factor Xa to convert prothrombin to thrombin by a factor of 278,000 and makes factor Xa refractory to inhibition by the anticoagulant, tissue factor pathway inhibitor. Protamine causes a dose-dependent decrease in factor V activation by α-thrombin, which accounts for most of the anticoagulant effect of protamine on the tissue factor pathway in human plasma; however, protamine has a small effect on plasma containing preformed factor Va. That mechanism for inhibition of thrombin generation was likely important in the delay in clot formation and decrease in clot strength caused by protamine in the present study.

The effects of protamine on platelets are poorly understood. Protamine can induce transient thrombo-
cy-topenia in dogs and humans that have or have not received heparin. Administration of protamine causes increased aggregation of platelets and thrombo-
cy-topenia in dogs, and results of an in vitro study indicate protamine decreases adenosine diphosphate–
induced platelet aggregation. In platelet-rich plasma, the effects of protamine on thrombin may be attenuated by platelets. Such attenuation of the effects of protamine by platelets may be a reason for the less substan-
tial thromboelastography coagulation effects detected in blood samples in the present study, compared with those detected in human plasma samples in another study. Although further studies are warranted, the interaction of protamine with platelets likely decreases platelet function and the effectiveness of platelets during clot formation. This likely contributed to the delay in clot formation and decrease in MA detected in blood samples with protamine in the present study.

Results of this study suggested that blood samples that are not activated with kaolin can be used to assess changes in coagulation caused by protamine. Small differences in results were detected between the blood samples activated with kaolin and those that were not activated. Further studies are warranted to determine which type of blood sample preparation is best for testing of clinical patients.

Results of the present study and those of other studies suggested that doses of protamine should be chosen carefully. When choosing a dose of protamine, the goal should be to achieve a physiologically normal state of coagulation in a patient. However, hypocoagu-
labile states may be worsened after administration of an inappropriate dose of protamine. Protamine is typically administered to dogs at doses up to 6 mg/kg. However, use of activated clotting times and heparin dose-
response curves may be appropriate for titration of protamine doses to circulating heparin concentrations. Results of the present study and those of similar studies for humans suggested such methods could reduce the risk of administration of an excessive dose of protamine and development of associated coagulopathies.

A major limitation of the present study was that it was conducted in vitro and the effects of endothelial cells on coagulation were not determined. Additionally, although clinically normal dogs were included in the study, complete blood analyses (including coagulation tests) were not performed for each dog. In future studies, the effects of heparin-protamine interactions on protamine-induced coagulopathies should be evaluated. In addition, the in vivo effects of protamine on coagulation as assessed with thromboelastography should be determined; in vivo effects of protamine on coagulation may differ from the in vitro effects determined in the present study.

Results of this study indicated protamine significantly reduced clot strength in canine whole blood samples as assessed by means of thromboelastography, and that effect seemed to be dose dependent. Results also suggested that doses of protamine for dogs should be chosen with caution.

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