The use of impedance aggregometry to evaluate platelet function after the administration of DDAVP in healthy dogs treated with aspirin or clopidogrel

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OBJECTIVE
To evaluate the effect of 1-Desamino-8-d-arginine vasopressin (DDAVP; desmopressin acetate) on platelet aggregation in healthy dogs receiving aspirin or clopidogrel.

ANIMALS
7 healthy staff-owned dogs.

PROCEDURES
In this randomized double-blinded crossover study, impedance aggregometry was performed on samples of lithium-heparinized whole blood samples from dogs before (T0) treatment with aspirin (1 mg/kg, PO, q 24 h for 4 days; ASP group) or clopidogrel (1 mg/kg, PO, q 24 h for 4 days; CLP group) and then before (T1) and after (T2) treatment with DDAVP (0.3 µg/kg, IV, once). There was a 14-day washout period before the crossover component. Aggregometry was performed with 4 different assays, each of which involved a different agonist reagent to stimulate platelet function: ADP, thrombin receptor activating peptide-6, arachidonic acid, or collagen type 1.

RESULTS
Median results for platelet aggregometry with agonist reagents ADP, arachidonic acid, or thrombin receptor activating peptide-6 significantly decreased between T0 and T1 for the CLP group; however, no meaningful difference in platelet aggregation was detected in the ASP group. Results for platelet aggregometry did not differ substantially between T1 and T2 regardless of treatment group or assay.

CONCLUSIONS AND CLINICAL RELEVANCE
Findings suggested that administration of DDAVP may have no effect on platelet aggregation (measured with platelet aggregometry) in healthy dogs treated with clopidogrel. Because no inhibition of platelet aggregation was detected for dogs in the ASP group, no conclusion could be made regarding the effects of DDAVP administered to dogs treated with aspirin.

Antiplatelet drugs, such as aspirin and clopidogrel bisulfate, are commonly used in people and dogs to prevent thromboembolism.[1] Aspirin inhibits cyclooxygenase, reducing platelet synthesis of TXA2, which is a potent platelet activation agonist; therefore, reduction of TXA2 with aspirin treatment inhibits platelet function.[1] Clopidogrel acts in vivo as a P_2Y_{12} platelet receptor antagonist, reducing platelet activation and thus platelet function.[1] Through inhibition of platelet function, these drugs also increase the risk for surgical and perioperative bleeding.[2–4] A treatment to quickly reverse the platelet inhibition action of these drugs would

be clinically relevant; however, evidence of useful treatments to reverse the effect of antiplatelet drugs in dogs is lacking.

Desmopressin acetate, also known as DDAVP, is a synthetic vasopressin analog used to decrease bleeding in dogs with von Willebrand disease by mediating the release of vWf from stores in the endothelium.[5,6] In people, DDAVP is also used to reverse the antiplatelet effects of aspirin and clopidogrel.[2,3] A single injection of DDAVP (0.3 to 0.4 µg/kg, IV) may improve platelet function, reverse the antiplatelet effects of aspirin and clopidogrel, and reduce perioperative bleeding in human patients taking these drugs.[2–4,7–10] Yet evaluations of DDAVP effects on platelet function or antiplatelet drug reversal in dogs are lacking. For instance, to our knowledge, assessment of BMBT (a crude assessment of primary hemostasis that is susceptible to intra- and interobserver variations[11]) in dogs receiving aspirin and given DDAVP is described in only 2

ABBREVIATIONS
BMBT	Buccal mucosal bleeding times
DDAVP	1-Desamino-8-d-arginine vasopressin (desmopressin acetate)
TRAP	Thrombin receptor activating peptide-6
TXA2	Thromboxane A2
vWf	von Willebrand factor

small case series,[12,13] and both show improvement in BMBT after a single injection of DDAVP. It is unknown whether DDAVP can improve platelet function or aggregation in dogs on antiplatelet drugs. If it can, DDAVP would be an attractive and widely available option to reduce bleeding risks in dogs on antiplatelet drugs. Rather than measuring BMBT, it would be ideal to use a more objective assessment of platelet function, such as platelet aggregometry, which is a useful tool to assess platelet function in dogs.[14] Platelet aggregometry can also be used to evaluate the efficacy of antiplatelet drugs such as aspirin and clopidogrel in dogs.[15,16]

The aim of the study reported here was to evaluate the effect of DDAVP on platelet aggregation in healthy dogs treated with aspirin or clopidogrel. We hypothesized that DDAVP administration to healthy dogs receiving aspirin or clopidogrel would result in greater platelet aggregation as assessed with impedance aggregometry.

Materials and Methods

This randomized double-blinded crossover study was performed at the University of Florida College of Veterinary Medicine. The study was approved by the College of Veterinary Medicine Hospital Research Review Committee and the University of Florida Institutional Animal Care and Use Committee (protocol No. 201609566).

On the basis of a previous study[15] of platelet inhibition induced by clopidogrel, a sample size calculation was performed and we determined that a sample size of 6 dogs would be required to reject the null hypothesis with a power of 0.8. Therefore, 7 healthy staff-owned dogs were recruited. Dogs were eligible if they had no abnormal findings on physical or hematologic examinations; were between 1 and 9 years of age; weighed > 4 kg; had not received other medications during the previous 2 weeks, except for parasite preventatives or dietary supplementations; and had no known allergic reactions to aspirin, clopidogrel, or DDAVP. Dogs were excluded if they had abnormal findings on physical examination; results for a baseline CBC, serum biochemical analyses, or platelet aggregometry outside of reference limits; adverse reaction to study medications; missing data during the study; or a noncompliant owner. All owners signed a consent form to participate in the study.

Study protocol

Dogs were randomly assigned to receive either aspirin (ASP group) or clopidogrel (CLP group), each at a dosage of 1 mg/kg, PO, daily for 4 days. Each drug was compounded into similar-appearing suspensions by a pharmacologist, and neither the primary investigators nor the owners were aware of which study drug the participating dogs received. Thus, this was a double-blinded study design. Whole blood samples were collected for baseline CBC, serum biochemical analyses, and platelet aggregometry[a] for each dog before the study

treatment was started (T0), and each dog served as its own control. The study treatments were administered as prescribed, and within 12 hours after receiving the antiplatelet medication on day 4, platelet aggregometry was performed again immediately before (T1) and 30 to 60 minutes after (T2) administration of DDAVP (4 μg/mL solution; 0.3 μg/kg, IV, once). There was a 14-day washout period before the crossover component of the study commenced, with each group then receiving the alternative treatment (aspirin or clopidogrel) for 4 days. The duration of the washout period was chosen according to the drugs' elimination half-lives and previous pharmacokinetic studies[15,17] of dogs. For the crossover component, all dogs underwent impedance aggregometry at T0 (baseline) and on day 4 before (T1) and after (T2) administration of DDAVP as described earlier.

Blood collection and DDAVP administration

For each dog at T0, a blood sample (approx 5 to 6 mL) was drawn from a jugular vein with a 20-gauge needle attached to a 6-mL syringe. The sample was collected after a single insertion into the vessel, minimizing premature platelet activation. Immediately after collection, each blood sample was divided among a set of evacuated collection tubes that consisted of 1 glass tube with no anticoagulant added (red top tube), 1 tube with EDTA added, and 1 tube with lithium heparin added, in that order, for serum biochemical analyses, a CBC, and platelet aggregometry, respectively. Immediately after the blood was transferred to collection tubes, the tubes with lithium heparin and EDTA added were carefully inverted 5 times to ensure adequate mixing of the contained blood and respective anticoagulant.

Immediately after T1 on day 4, each dog received DDAVP (0.3 μg/kg, IV), followed by phlebotomy 30 to 60 minutes later (T2). For each dog, this T2 sample was drawn from the contralateral jugular vein as described earlier. Only approximately 2 mL of blood was obtained at T1 and again at T2. Immediately after phlebotomy, each sample was transferred to an evacuated collection tube with added lithium heparin for later platelet aggregometry, and each tube was carefully inverted 5 times to ensure adequate mixing of the blood and lithium heparin.

Platelet aggregometry

The collection tubes containing lithium-heparinized blood were left in a standing position at room temperature (20 to 25 °C) until platelet aggregometry was performed between 30 and 180 minutes after sample collection according to the manufacturer's guidelines.[18] Evacuated collection tubes with lithium heparin added were chosen because a study[19] shows that lithium heparin is a superior anticoagulant for platelet aggregometry in dogs when performed with the aggregometer[a] we used.

Immediately before the volume of lithium-heparinized blood needed for platelet aggregometry analy-

sis was retrieved, the tube containing the given blood sample was inverted carefully 3 times. Each sample was evaluated with 4 separate assays, each of which involved a different reagent to stimulate platelet function: ADP,[b] TRAP,[c] arachidonic acid,[d] or collagen type 1.[e] Agonists were prepared and stored in aliquots of 150 µL at –80 °C for a maximum of 28 days and thawed at room temperature prior to analysis according to the manufacturer's guidelines. After use, the remaining aliquot of thawed agonist was discarded.

For each assay, the platelet aggregometer continuously recorded platelet aggregation for 6 minutes. The increase of impedance by the attachment of platelets onto the aggregometer's sensors was transformed to arbitrary aggregation units and plotted against time. The results for the area under the aggregation curve were affected by the total height and slope of the aggregation curve and were best suited to express the overall platelet activity. Results for each area under the aggregation curve were expressed as units (U) with the following formula: 1 U = 10 AU ✕ min, where AU was the aggregation unit. Results were reported as median units at T0, T1, and T2 for each treatment group.

On the basis of published criteria,[17,20] dogs in either the ASP or CLP group were considered to have been responders to the respective antiplatelet drugs if they had a ≥ 25% decrease in platelet aggregation results for ≥ 1 of the 4 assays at T1, compared with T0. A clinically relevant reversal of platelet function inhibition by DDAVP was defined empirically to have been a 50% improvement in platelet function results for ≥ 1 of the 4 assays at T2, compared with T1.

Statistical analysis

Data were assessed for normality with the D'Agostino-Pearson omnibus normality test. Median results for platelet aggregometry assays, designated by agonist reagent (ADP, TRAP, arachidonic acid, or collagen), were determined and evaluated for each group at T0, T1, and T2. The resulting amplitude and slope at T0, T1, and T2 were compared for the dogs in the ASP versus CLP group with repeated-measures 1-way ANOVA. The Friedman test with Dunn multiple comparisons test was used to compare the results between the ASP and CLP groups. To ensure that the washout period was effective, results for platelet aggregometry at T0 were compared between groups with the Wilcoxon signed-rank test. Statistical analysis was performed with available software[f]; values of $P < 0.05$ were considered significant.
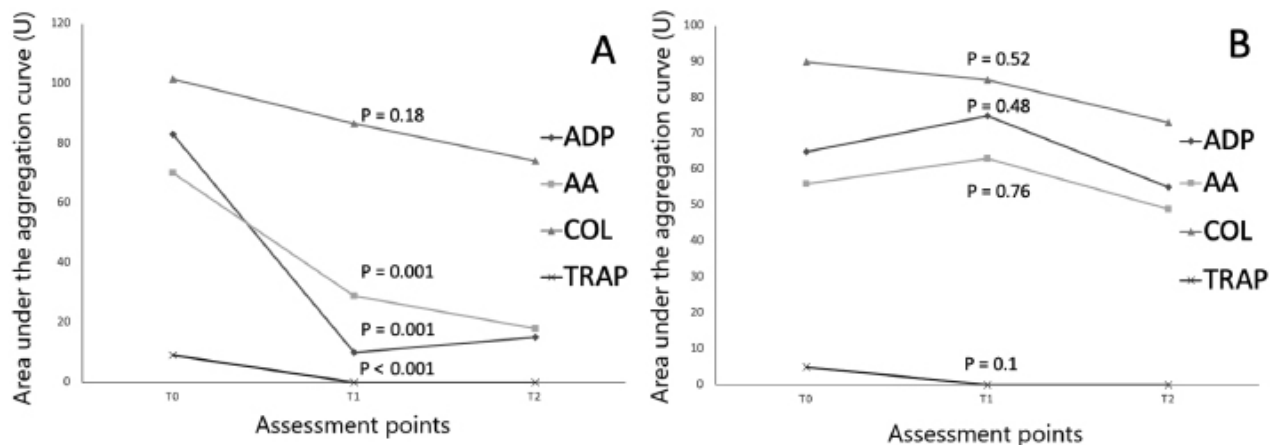
Results

Animals

Seven healthy staff-owned dogs (4 castrated males and 3 spayed females) were enrolled in the study. The median age was 4 years (range, 1 to 9 years), and the median body weight was 10.6 kg (range, 4.2 to 26.2 kg). The dogs included 5 mixed-breed dogs, 1 Shih Tzu, and 1 Border Collie. All dogs were deemed healthy on the basis of findings from physical examination, a CBC, serum biochemical analyses, and baseline platelet aggregometry.

Platelet aggregometry

The median results for baseline platelet function measured with impedance aggregometry at T0 did not substantially differ (ADP, $P = 0.938$; TRAP, $P = 0.688$; arachidonic acid, $P = 0.688$; and collagen, $P = 0.75$) between dogs in the CLP group (ADP, 65 U; TRAP, 5 U; arachidonic acid, 70 U; and collagen, 77 U) and the ASP group (ADP, 65 U; TRAP, 5 U; arachidonic acid, 56 U; and collagen, 91.5 U). For dogs in the CLP group, the median results of platelet aggregometry significantly decreased between T0 and

Figure 1—Results of impedance aggregometry with assays that used the agonist reagents ADP, TRAP, arachidonic acid (AA), or collagen type 1 (COL) to assess platelet function for dogs in the CLP group (A) and ASP group (B) before antiplatelet treatment (T0) and then before (T1) and after (T2) treatment with DDAVP. A—For dogs in the CLP group, the median results for the area under the aggregation curve as a measurement of platelet aggregation significantly decreased from T0 to T1 (ADP, $P = 0.001$; AA, $P = 0.001$; and TRAP, $P < 0.001$) but did not meaningfully change after administration of DDAVP (between T1 and T2) regardless of assay used. B—For dogs in the ASP group, there was no meaningful change in platelet aggregation detected between T0 and T1 or between T1 and T2, regardless of the assay used.

T1 on assays with the agonist reagent ADP (T0, 83 U; T1, 10 U; P = 0.001), arachidonic acid (T0, 70 U; T1, 29 U; P = 0.001), or TRAP (T0, 9 U; T1, 0 U; $P < 0.001$; **Figure** I). Dogs in the ASP group had no substantial differences in median results of any of the 4 assays between T0 and T1.

Responsiveness to antiplatelet effects of aspirin and clopidogrel

Four of the 7 dogs in the ASP group were considered to have been responders (ie, had a ≥ 25% decrease in platelet function detected by ≥ 1 of the platelet aggregometry assays). Among these 4 responders, 2 met the criteria of responsiveness on the basis of results of only the assay with TRAP as the agonist reagent, and both dogs had a 100% decrease in platelet function for T1, compared with T0. The remaining 2 responders in the ASP group had a ≥ 25% decrease in platelet function detected with ≥ 3 different types of assays **(Supplementary Table S1)**. All 7 dogs in the CLP group were considered to have been responders; however, when only results for the assay with collagen as the agonist reagent were considered, 2 dogs did not have a ≥ 25% decrease in platelet function between T0 and T1 and would not have been considered responders on the basis of results from this assay alone.

Platelet aggregometry after DDAVP administration

After administration of DDAVP, the median platelet aggregometry assay results were ADP, 15 U; TRAP, 0 U; arachidonic acid, 18 U; and collagen, 74 U for dogs in the CLP group and ADP, 55 U; TRAP, 0 U; arachidonic acid, 49 U; and collagen, 73 U for dogs in the ASP group (Figure 1). Results for platelet aggregometry did not differ substantially between T1 and T2 regardless of the treatment group or assay.

Discussion

Results of the present study indicated that DDAVP did not improve platelet aggregation in healthy dogs on clopidogrel treatment. Furthermore, the administration of aspirin in the present study did not provide a detectable amount of platelet inhibition as assessed by multiple-electrode impedance aggregometry, which in turn prevented meaningful evaluation of the effects of DDAVP administration in dogs receiving aspirin.

In contrast to our findings in healthy dogs, studies in human medicine[8,21-23] show that healthy people receiving aspirin, cyclooxygenase-1 inhibitors, or ADP receptor inhibitors have improvement in several tests of platelet function when treated with versus without DDAVP; however, these findings have not directly translated to associations with clinical outcomes. Also, in human medicine, a recent guideline[24] recommends to discontinue antiplatelet agents and consider a platelet product transfusion and single IV injection of DDAVP for reversal of antithrombotics

in people with intracranial hemorrhage; yet controversy remains. For instance, a study[25] shows that the use of DDAVP significantly reduced blood loss and improved thrombus formation in patients undergoing cardiac surgery and that had been exposed to aspirin preoperatively. Conversely, results of 2 randomized double-blind trials[26,27] indicate that the use of DDAVP in patients undergoing cardiopulmonary bypass or aortic surgery had no identified benefit.

Another important finding of our study was that the administration of clopidogrel at the dosage of 1 mg/kg, PO, once daily for 4 days resulted in a substantial decrease in platelet function in all dogs such that all dogs were considered to have been responders, whereas only 4 of the 7 dogs were considered responders to the antiplatelet effect of aspirin when it was administered at the same dosage. Unfortunately, treatment with low dose aspirin (0.5 to 1 mg/kg, PO, q 24 h), unlike high dose aspirin (5 to 10 mg/kg, PO, q 24 h), does not consistently inhibit platelet function in dogs.[17,28,29] In human medicine, patients who are poorly responsive to the antiplatelet effects of aspirin are termed aspirin resistant.[30] The incidence rates of low-dose aspirin resistance range from 5% (17/326)[30] to 60% (42/70)[31] in humans and 19% (3/16)[17] to 33% (8/24)[28] in healthy dogs, depending on the technique used to assess inhibition of platelet function. Information varies regarding the appropriate aspirin dose to inhibit platelet function in dogs. For example, McLewee et al[32] reported that, for 8 healthy dogs, the minimum aspirin dosage to consistently result in aspirin responsiveness was 2 mg/kg, PO, daily, which is higher than the dose used in our study. Thus, a higher aspirin dose (at least 2 mg/kg, PO, q 24 h) could be considered to improve the aspirin response rate in dogs for future research.

There are many reasons why aspirin might not suppress the production of TXA2 and aggregation of platelets, consistent with aspirin resistance in laboratory settings. These reasons include compliance; dosage; altered absorption, metabolism, or both; the presence of nonplatelet sources of TXA2 production; other pathways of platelet activation; increased platelet turnover; genetic polymorphisms; and loss of the antiplatelet effect of aspirin with prolonged administration (tachyphylaxis).[30]

Our study had several limitations. There is no certainty that the results of the present study would be reproduced in dogs with naturally occurring diseases in which platelet function may be affected by a variety of disease processes. Similar to human research, experimental data obtained from healthy volunteers may differ from clinical patients. That being said, it is important to document that DDAVP works in healthy dogs first before it can be used in clinical trials.

The next limitation was our use of impedance aggregometry to assess platelet function. Optical aggregometry is the gold standard modality for the assessment of platelet function in animals and humans[33]; however, it is extremely cumbersome. Results from

impedance aggregometry have good agreement and correlation with optical aggregometry[34]; therefore, we believe it is likely that similar results would have been found with optical aggregometry. In studies[22,24] of human patients, the effects of DDAVP on platelet function were assessed with a point-of-care device that evaluates primary hemostasis with a high-shear force dynamic flow system, which is more sensitive than the skin bleeding time for detection and monitoring of von Willebrand disease. Consideration for future research could include comparative platelet function assessments with impedance aggregometry versus high-shear force dynamic flow system.

Another limitation was that some veterinary studies[17,35] that indicate platelet aggregometry may have poor sensitivity in detecting platelet inhibition caused by aspirin administration in dogs involved the use of different anticoagulants for whole blood samples and different agonist reagents, making the direct comparison difficult. However, Mueller et al[36] show that the platelet aggregometer is useful for specific detection of both aspirin and clopidogrel effects on platelet function in people. In that study,[36] the authors applied the test-specific reference limits as criteria for differentiation between responders and nonresponders to aspirin treatment, whereas we used the cutoff of a 25% reduction in platelet function as a main criterion of responsiveness to platelet inhibitors. The use of different criteria of responsiveness as well as various combinations of reagents could potentially explain the differences in findings.

Additionally, it was possible that the administration of DDAVP to the dogs of the present study did not stimulate the release of vWf, which cannot be proven without measurement of vWf concentration. Thus, measurements of vWf antigen concentration could have theoretically improved the monitoring of DDAVP effects, given its main mechanism of action is an enhanced release of vWf from the endothelial stores.[5,6] In addition, our study was not designed to assess an improvement in platelet adhesion caused by increased levels of vWf. Therefore, we cannot definitively claim that DDAVP did not improve platelet function without the measured vWf levels or evaluation of the platelet adhesion. Conversely, there is evidence in human medicine that suggests the existence of physiologic mechanisms by which DDAVP improves platelet function without an increase in plasma concentration of vWf.[37-39]

Furthermore, we recognize the possibility of poor compliance by owners of dogs in the present study because the platelet inhibitors were administered at home. Poor owner compliance could have resulted in fewer dogs with responsiveness to the aspirin treatment. However, poor compliance was considered unlikely because the owners were blinded to the study drug and all dogs responded to the clopidogrel treatment.

In conclusion, findings from the present study suggested that administration of DDAVP to healthy dogs treated with clopidogrel for 4 days had no reversal effects on platelet function inhibition as assessed by impedance aggregometry. Treatment with clopidogrel (1 mg/kg, PO, q 24 h for 4 days) reduced platelet function $\geq 25\%$ between T0 and T1 in all 7 dogs, whereas only 4 of 7 dogs responded (had reduced platelet function $\geq 25\%$ between T0 and T1) to treatment with aspirin at the same dosage.

Footnotes

a. Multiplate Analyzer, Roche Diagnostics GmbH, Mannheim, Germany.
b. Multiplate ADPtest Kit, Roche Diagnostics GmbH, Mannheim, Germany.
c. Multiplate TRAPtest Kit, Roche Diagnostics GmbH, Mannheim, Germany.
d. Multiplate ASPItest Kit, Roche Diagnostics GmbH, Mannheim, Germany.
e. Multiplate COLtest Kit, Roche Diagnostics GmbH, Mannheim, Germany.
f. Prism, version 5.0, GraphPad Software, San Diego, Calif.

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Supplementary Materials

Supplementary materials are available online at: avmajournals.avma.org/doi/suppl/10.2460/ajvr.82.10.823.