Platelet aggregation in dogs with mitral valve regurgitation

Ryou Tanaka, DVM, and Yoshihisa Yamane, DVM, PhD

Objective—To compare platelet aggregation in healthy dogs and dogs with mitral valve regurgitation (MVR) to determine whether regurgitation had an effect on platelet function.

Animals—32 dogs with MVR and 43 healthy dogs.

Procedure—Platelet aggregation was measured with an aggregometer, using adenosine 5′-diphosphate as the aggregating agent, and the maximum aggregation and the enhancement of platelet sensitivity (EPS) values were calculated.

Results—Platelet count and maximum aggregation were not significantly different between healthy dogs and dogs with MVR. However, EPS values in dogs with MVR were significantly higher than values in healthy dogs. Platelet count and maximum aggregation were not significantly different between dogs classified as New York Heart Association functional class I or II and dogs classified as functional class III or IV; however, EPS values were significantly higher in dogs classified as functional class III or IV.

Conclusions and Clinical Relevance—Results suggest that platelet aggregation is decreased in dogs with MVR and that the EPS value may be more sensitive to differences in disease severity than in measurement of maximum aggregation. (*Am J Vet Res* 2000;61:1248–1251)

Aggregation of platelets in the small arteries is the first step in arterial thrombosis. In humans, platelet microthrombi may occlude the capillaries of the cardiac musculature, resulting in arrhythmia,[1] and nontransmural myocardial infarction that is not associated with coronary thrombosis may be a result of platelet microthrombi. Thromboembolic complications of mitral valve disease have been reported, and these complications are suspected to be mediated by platelets.[2-6] Degeneration, necrosis, and fibrosis of the cardiac musculature is common in dogs with **mitral valve regurgitation (MVR)**, suggesting that platelets may play a role.[7-10] However, there are few reports investigating the mechanism of microthrombus formation and related complications in dogs with MVR. The purpose of the study reported here was to compare platelet aggregation in healthy dogs and dogs with MVR to determine whether regurgitation had an effect on platelet function.

There is no standard method of evaluating platelet aggregability, and test procedures and aggregating substances vary from one institute to the next. It is thought that the threshold concentration for irreversible aggregation is the best way to evaluate platelet aggregability, but maximum aggregation can be used as a substitute. Recently, a new method of evaluating platelet aggregability was reported.[11] The result of this method is called the **enhancement of platelet sensitivity (EPS)**, or the platelet aggregatory threshold index, and the threshold concentration for irreversible aggregation can easily be measured by use of this method. The EPS value also reflects platelet sensitivity more accurately than do standard measures in human patients with cerebral infarction.[12] Therefore, in the present study, we compared EPS values with maximum aggregation.

Materials and Methods

Evaluation of reproducibility—Five healthy dogs (2 males and 3 females; 4 Beagles and 1 mixed-breed dog) were used to evaluate the reproducibility of platelet counts and measurements of EPS values and maximum aggregation. Dogs ranged from 0.62 to 3 years old (mean ± SEM, 1.23 ± 0.44 y) and weighed between 7 and 12 kg (9.9 ± 2.0 kg).

Six blood samples were collected from each dog on separate days. Because blood collection and handling techniques can affect platelet aggregation, a standard method of blood collection was used.[13,14] Dogs were not sedated or anesthetized during blood collection; the only restraint was physical restraint by experienced staff members. Jugular vein blood samples were collected with plastic disposable syringes connected to 22-gauge needles coated with 3.8% trisodium citrate.[a] Blood samples were mixed in plastic tubes with 3.8% trisodium citrate at a concentration of 9 volumes of blood to 1 volume of trisodium citrate. Samples were maintained at a constant room temperature (22 C) so as not to induce unnecessary stimulation, and platelets were separated by means of centrifugation at 150 × g for 10 minutes.[15] The **platelet-rich plasma (PRP)** was transferred by careful pipetting with plastic pipettes into plastic containers. The packed cells were then centrifuged at 2,000 × g for 30 minutes, and the **platelet-poor plasma (PPP)** was harvested.

Comparison of platelet aggregability in healthy dogs and dogs with MVR—Blood samples were collected from 11 healthy dogs without heart disease examined at the Tokyo University of Agriculture and Technology Veterinary Teaching Hospital for a variety of reasons (vaccination, n = 3; castration, 3; physical examination, 3; ear cleaning, 1; and ovariohysterectomy, 1) and from 27 healthy Beagles. Dogs did not have evidence of any disease that could affect platelet function, such as a malignant tumor or diabetes mellitus. Of the 38 dogs, 24 were male, and 14 were female. Dogs ranged from 0.46 to 12.84 years old (3.47 ± 0.62 y) and weighed between 1.6 and 40.4 kg (10.3 ± 1.2 kg).

In addition, blood samples were collected from 32 dogs (21 males and 11 females) in which MVR had been diagnosed at the Tokyo University of Agriculture and Technology Veterinary Teaching Hospital. The diagnosis had been made on the basis of results of auscultation, radiography, and ultra-

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From the Department of Veterinary Surgery and the Tokyo University of Agriculture & Technology Veterinary Teaching Hospital, Tokyo University of Agriculture & Technology, 3-5-8 Saiwai-cho Fuchu-shi, Tokyo, Japan.
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sonography. Enalapril maleate (0.25 mg/kg/day) was prescribed in 5 dogs, and a combination of enalapril maleate (0.25 mg/kg/day) and digoxin (0.05 mg/kg/day) was prescribed in 4 dogs. No antiplatelet drug was prescribed. Twelve dogs were classified as **New York Heart Association (NYHA)** functional class I or II, and 20 dogs were classified as NYHA functional class III or IV.[16-18] Dogs ranged from 6.9 to 19.0 years old (12.6 ± 0.5 y) and weighed between 1.5 and 29.0 kg (8.4 ± 1.1 kg).

Determination of platelet aggregation—Platelet aggregation was measured by the change in light transmission, as described.[15] Glass cuvettes (6 × 45 mm) produced specifically for measurement of platelet aggregation and containing a magnet stirrer bar[b] were used. Two hundred microliters of PPP was placed in a cuvette for calibration. Platelet count of the PRP samples was adjusted to 300,000 to 400,000/µl, using fresh autologous PPP, and 100 µl was transferred to each of at least 4 cuvettes. Platelet aggregation was measured 90 minutes after blood collection by use of an aggregometer,[c] which continuously records light transmission after addition of aggregating agents. The aggregating agent used was **adenosine 5′-diphosphate (ADP)**.[d]

A 1-m*M* stock solution of ADP was made with isotonic saline solution, and 0.5-ml aliquots were stored at –30 C for daily use. Each day, aliquots of the stock ADP solution were thawed, and stepwise dilutions ranging from 800 to 6.25 µ*M* were created; diluted solutions were kept on ice until used. The PRP was warmed to 37 C for 1 minute, and 11 µl of the diluted ADP solution was added, so that final concentration of ADP ranged from 80 to 0.625 µ*M*. For each ADP concentration, the change in light transmission at 5 minutes was obtained and plotted as change in light transmission versus ADP concentration. From this curve, the EPS value was obtained.[11,12] The EPS value was defined as the concentration of ADP that evoked 50% of the irreversible maximum aggregation. Maximum aggregation was defined as the extent of aggregation within 7 minutes after addition of 50-µ*M* ADP (final concentration of 5 µ*M*). Platelet counts were determined by use of the Brecher-Cronkite method.[19]

Statistical analyses—The Mann-Whitney *U*-test was used to determine whether platelet count, maximum aggregation, or EPS value were significantly different between healthy male and healthy female dogs and between male dogs with MVR and female dogs with MVR. The correlation coefficient (R^2) was used to determine, for healthy dogs and dogs with MVR, whether the EPS value was correlated with age. Platelet count, maximum aggregation, and EPS value were compared between healthy dogs and dogs with MVR by means of the Mann-Whitney *U*-test. In addition, the Mann-Whitney *U*-test was used to compare platelet count, maximum aggregation, and EPS values for dogs that were NYHA functional class I or II with values for dogs that were functional class III or IV. All analyses were performed with statistic software.[e] Values of $P < 0.05$ were considered significant.

Results

Reproducibility of measurements—Platelet counts measured 6 times in 5 healthy dogs ranged from 31.5 to 62.33 × 10^4/µl (mean ± SEM, 40.38 ± 1.74 × 10^4/µl), EPS values ranged from 3.48 to 6.60 µ*M* (4.73 ± 0.35 µ*M*), and maximum aggregation ranged from 35.5 to 61.33% (52.57 ± 3.12%). Coefficients of variation for platelet counts, EPS, and maximum aggregation were 2.28, 4.11, and 3.25, respectively.

Comparison of platelet aggregability in healthy dogs and dogs with MVR—For the 38 healthy dogs, mean ± SEM platelet count was 41.9 ± 1.8 × 10^4/µl, mean EPS value was 5.31 ± 0.35 µ*M*, and mean maximum aggregation was 51.5 ± 3.8%. Platelet count, EPS value, and maximum aggregation were not significantly different in healthy female dogs versus healthy male dogs ($P = 0.116$, 0.053, and 0.087, respectively). Age was not significantly correlated with EPS value ($R^2 = 0.040$).

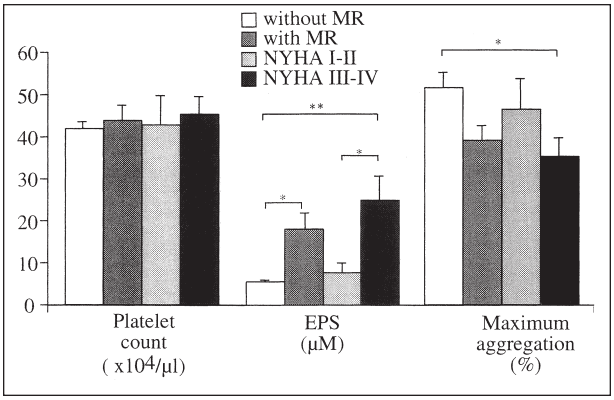

Figure 1—Mean platelet counts, enhancement of platelet sensitivity values, and maximum platelet aggregation in 38 healthy dogs and 32 dogs with mitral valve regurgitation ([MR]; 12 classified as New York Heart Association [NYHA] functional class I or II and 20 classified as NYHA functional class III or IV). Error bars represent SEM. *Values were significantly ($P < 0.05$) different. **Values were significantly ($P < 0.01$) different.

For the 32 dogs with MVR, mean ± SEM platelet count was 43.8 ± 3.7 × 10^4/µl, mean EPS value was 18.01 ± 4.05 µ*M*, and mean maximum aggregation was 38.9 ± 3.9%. Platelet count ($P = 0.732$) and maximum aggregation ($P = 0.074$) were not significantly different between healthy dogs and dogs with MVR. However, EPS values in dogs with MVR were significantly ($P = 0.017$) higher than values in healthy dogs (**Fig 1**). Platelet count, EPS value, and maximum aggregation were not significantly different in female dogs with MVR versus male dogs with MVR ($P = 0.056$, 0.513, and 0.797, respectively). Age was not significantly correlated with EPS value ($R^2 = 0.013$).

Platelet count ($P = 0.683$) and maximum aggregation ($P = 0.350$) were not significantly different between dogs classified as NYHA functional class I or II and dogs classified as functional class III or IV; however, EPS value was significantly ($P = 0.010$) higher in dogs classified as functional class III or IV (Fig 1). Maximum aggregation was significantly ($P = 0.021$) lower and EPS value was significantly ($P = 0.001$) higher for dogs classified as functional class III or IV than for healthy dogs. Platelet count ($P = 0.571$), maximum aggregation ($P = 0.811$), and EPS value ($P = 0.426$) for dogs classified as functional class I or II were not significantly different from values for healthy dogs.

Discussion

Measurement of the extent of platelet aggregation is the best method of determining platelet aggregability. Platelet aggregation can be semiquantitatively measured by continuously recording changes in light transmission after an aggregating agent has been added to PRP.[15] Various attempts have been made to interpret the resulting curves according to mass action laws, and various parameters have been devised to express aggre-

gability. Platelet aggregation is affected by sensitivity to the aggregating agent; therefore, the minimum concentration of the aggregating agent that evokes irreversible aggregation must be determined. However, determining the threshold concentration of an aggregating agent that evokes irreversible aggregation requires a large volume of blood, so that several concentrations of the aggregating agent can be tested. In addition, assessment of the aggregation curve is difficult because of subjective interpretation and incompatibility of results. The EPS value, on the other hand, provides uniform individual measurements and clearly distinguishes abnormal from normal values. In the present study, EPS values of dogs classified as NYHA functional class I or II were significantly different from values of dogs classified as functional class III or IV, suggesting that the EPS value is more sensitive to differences in disease severity than maximum aggregation.

Many factors influence platelet aggregability, including the standard of assessment, duration of centrifugation, temperature of samples, and interval from blood collection to analysis.[14,15] Thus, platelet aggregation should be evaluated under constant conditions. In the present study, careful attention was paid to adhering to a standard evaluation procedure to avoid errors in measurement. In particular, blood samples were collected and analyzed, using the same instruments, equipment, and methods, and anxious or excited dogs were excluded from the study. In humans, large-size (eg, 18 gauge) needles are used for blood collection to minimize any effect on the platelets.[18] However, an 18-gauge needle is not suitable for small dogs, so 22-gauge needles were used in the present study. During blood collection, aspiration with negative pressure was avoided to prevent any untoward effects on platelet integrity. Because dogs included in the study were client owned, a limited amount of blood was collected, and only a few laboratory tests were conducted.

There was a significant difference in mean age of healthy dogs and dogs with MVR in the present study. Breddin[20] reported that platelet aggregability was higher in people > 50 years old, compared with younger individuals. In the present study, EPS values for healthy dogs were not significantly correlated with age, but healthy dogs in the study were generally young, and whether age has an effect on EPS values in older dogs was not determined.

Aggregating agents other than ADP that can be used to measure platelet aggregability include collagen, platelet activating factor, and arachidonic acid. However, in the present study, only ADP was used, because the technique for determining EPS values requires measurements with at least 4 concentrations of ADP. Other methods of evaluating platelet function in humans include the platelet adhesion test, platelet releasing test, measurement of platelet lifespan, and determination of serum concentrations of β-thromboglobulin or platelet factor 4.[1,3,5,21]

Conflicting results have been reported regarding platelet function in human patients with heart disease. In some studies of human patients with chronic heart disease,[1,5,22] platelet activation and destruction were observed, whereas in others,[6,23,24] platelet hypoaggregability and a short platelet life span were detected. McGill et al[25] reported that a patient with congenital heart failure had larger platelets than normal and abnormally fast aggregation after addition of epinephrine. Bleeding tendencies have been observed in patients with congenital heart disease,[26] and in some patients with congenital heart disease, platelet counts were normal, but the platelets lacked normal aggregation.[26-29] Together, these studies suggest that platelets may play some role in the pathogenesis of chronic heart disease in humans. On the other hand, few clinical studies of platelet function in dogs and cats with or without heart disease have been published.[30-33]

In the present study, the lower platelet aggregability in dogs with severe MVR suggests that regurgitation at the mitral valve may expose platelets to continuous stimulation and stress, leading to exhaustion of platelets. Structural changes in platelets from human patients with heart disease have been reported,[24,34] and the same changes can occur in dogs with heart disease. Most studies involving humans suggest that stimulation secondary to MVR results in platelet hyperaggregability. However, results of the present study indicate that the opposite is true, and that rather than an enhancement of platelet function, a decrease in platelet function was observed in dogs with severe MVR (ie, NYHA functional class III or IV). In dogs with mild MVR (ie, NYHA functional class I or II), a decrease in platelet aggregability was not observed. This may suggest that in dogs with mild MVR, regurgitation is not so severe as to cause injury to platelets.

There are many tests that can be used to evaluate platelet function in vitro, but there is no ideal test that can be used to evaluate platelet function in vivo. In this study, the EPS value was used to evaluate platelet aggregability and was found to be low in dogs with MVR. Formation of microthrombi and fibrosis of the heart muscle in dogs with MVR, therefore, cannot be explained on the basis of changes in EPS value. However, in dogs with MVR, abnormal platelet function could still play a role in the pathogenesis of the disease. Detailed evaluation of EPS values and of results of other tests of platelet function at each stage of the disease will give additional information on the nature of platelet abnormalities in dogs with MVR.

[a]Citral for Sedimentation, Yamanouchi Pharmaceutical Co Ltd, Tokyo, Japan.
[b]SSR3111, SSR Engineering Co Ltd, Tokyo, Japan.
[c]MEBA 2 (model PAM-6C), SSR Engineering Co Ltd, Tokyo, Japan.
[d]Adenosine 5′-diphosphate sodium salt from equine muscle, Sigma Chemical Co, St Louis, Mo.
[e]Stat View, version 4.5, Abacus Concepts, Berkeley, Calif.

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