Effects of sodium citrate, low molecular weight heparin, and prostaglandin E_1 on aggregation, fibrinogen binding, and enumeration of equine platelets

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Objective—To investigate the effects of sodium citrate, low molecular weight heparin (LMWH), and prostaglandin E_1 (PGE_1) on aggregation, fibrinogen binding, and enumeration of equine platelets.

Sample Population—Blood samples obtained from 4 Thoroughbreds.

Procedure—Blood was collected into syringes in the ratio of 9 parts blood:1 part anticoagulant. Anticoagulants used were sodium citrate, LMWH, sodium citrate and LMWH, or 300 n*M* PGE_1/ml of anticoagulant. Platelet aggregation in response to ADP, collagen, and PGE_1 was assessed, using optical aggregometry. Platelet activation was evaluated, using flow cytometry, to detect binding of fluorescein-conjugated anti-human fibrinogen antibody. Plasma concentration of ionized calcium was measured, using an ion-selective electrode.

Results—Number of platelets (mean ± SEM) in samples containing LMWH ($109.5 \pm 11.3 \times 10^3$ cells/µl) was significantly less than the number in samples containing sodium citrate ($187.3 \pm 30.3 \times 10^3$ cells/µl). Increasing concentrations of sodium citrate resulted in reductions in platelet aggregation and plasma concentration of ionized calcium. Addition of PGE_1 prior to addition of an agonist inhibited platelet aggregation in a concentration-dependent manner, whereas addition of PGE_1 4 minutes after addition of ADP resulted in partial reversal of aggregation and fibrinogen binding.

Conclusions and Clinical Relevance—A high concentration of sodium citrate in blood samples decreases plasma concentration of ionized calcium, resulting in reduced platelet aggregation and fibrinogen binding. Platelets tend to clump in samples collected into LMWH, precluding its use as an anticoagulant. Platelet aggregation and fibrinogen binding can be reversed by PGE_1, which may result in underestimation of platelet activation. (*Am J Vet Res* 2001; 62:547–554)

Interest has been growing in the assessment of function for equine platelets. Studies reveal that platelet activation may play a role in the pathophysiologic process of laminitis[1] and contribute to thrombocytopenia seen in horses with equine infectious anemia.[2] In addition, platelets may be activated during exercise.[3,4] However, although there is evidence of activation of equine platelets in association with exercise, results of studies have been inconsistent. In early studies, it was reported that there was reduced platelet aggregation immediately following strenuous exercise.[5-7] In a study by Weiss et al,[3] there was an increased proportion of neutrophil-platelet aggregates following exercise, although a direct increase in platelet activation was not documented.

Several methods are available for the assessment of platelets, including platelet aggregation, measurement of plasma concentration of platelet secretory products, and assessment of binding of antibodies to specific platelet markers by use of flow cytometry. Each of these techniques has limitations. However, flow cytometry is now the most widely used technique in studies of human platelets.[8-10] Unfortunately, there is often poor species crossreactivity with many of the antibodies used to assess platelets. A goat anti-human fibrinogen antibody has been used to measure activation of equine platelets.[2,11] However, to the authors' knowledge, it is the only antibody for use in measuring activation of equine platelets that has been reported.

Results of tests that measure platelet function are subject to variation because of the methods used and possible in vitro activation of platelets secondary to handling procedures. The choice of anticoagulant can substantially influence results. For example, unfractionated heparin causes activation and aggregation of human platelets.[12-14] Sodium citrate chelates calcium and is routinely used as an anticoagulant in studies of platelets. However, changes in Hct of blood samples impact the plasma concentration of sodium citrate, which is typically added in the ratio of 1 part sodium citrate:9 parts blood and, therefore, the ionized calcium concentration in plasma samples.[15] Because calcium is important in platelet aggregation and the binding of fibrinogen,[16,17] it is possible that fibrinogen binding may be reduced in samples with a high Hct that are collected into sodium citrate. We are not aware of any information that addresses the possible effect of sodium citrate concentration on fibrinogen binding and its detection with flow cytometry.

Low molecular weight heparin (LMWH) has been used as an anticoagulant for in vitro studies of human platelets.[18] Enoxaparin sodium, a LMWH, did not cause activation of platelets in control samples evaluated by use of flow cytometry[19]; similarly, a 10-fold

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increase in the concentration of LMWH in blood samples obtained from horses did not alter platelet aggregation responses.[4] Therefore, LMWH may be a viable alternative as an anticoagulant for use when assessing platelets in samples in which the Hct is greater than the upper limit of the reference range (45 to 50%). However, we are not aware of any published reports pertaining to the effect of LMWH on fibrinogen binding to platelets. Such information is needed to determine the usefulness of LMWH as an alternative anticoagulant for samples in which investigators will be assessing equine platelets.

Concerns about in vitro activation of platelets have prompted researchers to add platelet inhibitors such as **prostaglandin E_1 (PGE_1)** to anticoagulants to limit activation of platelets by handling procedures.[11,20,21] However, the impact PGE_1 may have on measures of platelet function was not considered in those studies. Janes et al[22] documented that PGE_1 reversed fibrinogen binding in a concentration-dependent manner in preparations of human platelets. In addition, Welles et al[23] reported that PGE_1 caused reversal of aggregation in feline platelets and reduced binding of fibrinogen. Such reports raise questions about results obtained from studies in which antifibrinogen antibody was used to measure platelet activation in samples collected into sodium citrate and PGE_1. Therefore, the objective of the study reported here was to investigate the effect of sodium citrate, LMWH, and PGE_1 on aggregation, fibrinogen binding, and enumeration of equine platelets.

Materials and Methods

Animals—Four healthy sedentary Thoroughbreds ranging from 5 to 18 years old were used in the study. All procedures were conducted in compliance with guidelines established by the Washington State University Animal Care and Use Committee.

Collection of samples—Blood samples were collected by venipuncture, using an 18-gauge needle. Samples were collected once from each horse, but all anticoagulants and conditions were used for samples obtained from each of the 4 horses.

Samples were collected into syringes containing anticoagulant (9 parts blood:1 part anticoagulant). Anticoagulants used were 0.11*M* sodium citrate,[a] 200 U of LMWH[b]/ml, a combination of 0.11*M* sodium citrate and 200 U of LMWH/ml (final concentrations in the combined anticoagulant), or 300 n*M* PGE_1[c]/ml of anticoagulant. In some samples, concentration of sodium citrate or LMWH was adjusted. Concentration of anticoagulant in the original sample was calculated on the basis of the Hct of the sample, and additional anticoagulant then was added to increase the concentration of anticoagulant such that the final concentration was equivalent to that found in a blood sample with a Hct of 60%. The Hct for each sample was determined, using the microhematocrit method.

Preparation of platelet-rich plasma—Platelet-rich plasma (PRP) was prepared at room temperature (21 to 22 C) by centrifuging blood samples (150 × g for 10 minutes). **Platelet-poor plasma (PPP)** was prepared by centrifuging blood at 2,000 × g for 10 minutes. Platelet counts were performed on PRP, using an automated counter.[d]

Assessment of platelet aggregation—Platelet aggregation was performed with a dual-channel optical aggregometer[e] connected to a computer via an interface.[f] Platelet counts of samples containing LMWH and sodium citrate ranged from 98,000 to 138,000 cells/µl and 146,000 to 254,000 cells/µl, respectively. Levine[24] and Brackett et al[25] reported that platelet aggregation responses did not differ in samples with platelet counts ranging from 100,000 to 300,000 cells/µl. Therefore, because all samples had platelet counts < 300,000 cells/µl, none of the platelet counts was adjusted by use of PPP.

Samples of PRP were stirred at 37 C for 1 minute before the addition of agonist. Aggregation responses were evaluated by adding various concentrations (2.5, 1.25, 0.625, and 0.3125 µ*M*) of ADP[g] and various concentrations (4, 2, and 1 mg/µl) of collagen.[h] Frozen aliquots of ADP were thawed immediately prior to use and diluted in isotonic saline (0.9% NaCl) solution, as indicated. The platelet aggregation response was monitored for 5 minutes.

To assess the effect of PGE_1 on aggregation, PGE_1 was added to PRP (final concentration in PRP of 10^{-8} to $10^{-6}M$) before or 4 minutes after the addition of 2.5 µ*M* ADP or 4 µg of collagen/ml, and the aggregation response was monitored for an additional 10 minutes. To assess the effect of citrate on aggregation, sodium citrate (calculated concentration in samples with Hct of 35, 50, and 60%) was added 4 minutes after addition of ADP or collagen to samples containing LMWH, and the aggregation response was monitored for an additional 10 minutes. Platelet aggregation responses were assessed on the basis of amplitude and initial slope of the aggregation curve.

Assessment of platelet activation—Platelets were activated by adding ADP or collagen to PRP to achieve a final concentration of 2.5 µ*M* ADP or 4 µg of collagen/ml. Samples then were incubated for 15 minutes at room temperature (21 to 22 C). To assess the effect of PGE_1 on detection of platelet activation, PGE_1 (final concentration of 10^{-8} to $10^{-6}M$) was added to samples previously incubated with ADP or collagen, and aliquots were obtained for analysis 10 minutes after addition of PGE_1. The effect of sodium citrate was assessed by collecting 20 µl of PRP at the completion of each aggregation study, as described previously. Each 20-µl aliquot of PRP was diluted in 100 µl of PBS solution and fixed overnight in 1 ml of a freshly prepared 1% solution of paraformaldehyde. Fixed platelets were centrifuged at 1,000 × g for 2 minutes, the supernatant was discarded, and the pellet was resuspended in 500 µl of 0.5% **bovine serum albumin (BSA)** in modified Tyrode solution plus 500 µl of tris NH_4Cl. Platelets then were washed once in 0.5% BSA in modified Tyrode solution and centrifuged at 1,000 × g for 2 minutes. The pellet was resuspended in 500 µl of 0.5% BSA in modified Tyrode solution containing a fluorescein-conjugated goat $F(Ab')_2$ fragment to human fibrinogen antibody[i] at a dilution of 1:500. Samples were incubated for 20 minutes in the dark and then washed in 0.5% BSA in modified Tyrode solution before analysis. Negative-control samples were incubated with a fluorescein-labeled goat $F(Ab')_2$ fragment to mouse IgG.[j]

Using a flow cytometer,[k] the platelet population was identified by forward and side-angle scatter, the platelet cloud was identified, and 10,000 gated events were collected for each sample. Mean fluorescence of the population was used to assess the extent of platelet activation.

Measurement of plasma concentration of ionized calcium—Aliquots of PPP for each sample were stored at –80 C until analysis. Plasma concentration of ionized calcium was measured, using an ion-selective electrode.[l] Repeated standard curves were generated for calcium concentrations over the range of 10^{-5} to $10^{-2}M$, using highly purified $CaCl_2$ in solutions that were the ionic strength of plasma (140 m*M* NaCl, 3.2 m*M* KCl, 1.0 m*M* $MgCl_2 \cdot 6H_2O$). On a logarithmic scale, the electrode response was linear for concentrations

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Table 1—Hematocrit, platelet count, and plasma concentration of ionized calcium in blood samples the horses weren't collected into sodium citrate (citrate), a combination of sodium citrate and prostaglandin E_1 (PGE_1), sodium citrate adjusted to the calculated concentration in a sample with a hematocrit of 60% (citrate Hct 60), low molecular weight heparin (LMWH), a combination of LMWH and PGE_1, LMWH adjusted to the calculated concentration in a sample with a hematocrit of 60% (LMWH Hct 60), and a combination of sodium citrate and LMWH

Sample	Hct (%)	Platelet count ($\times 10^3$ cells/µl)	Ionized calcium (m*M*)
Citrate	31.5 ± 3	187.3 ± 30.5	$75 \pm 5.6 \times 10^{-3}$
Citrate and PGE_1	ND	187.5 ± 27.9	ND
Citrate Hct 60	ND	ND	$30.4 \pm 3.6 \times 10^{-3}$*
LMWH	31.3 ± 2.8	109.5 ± 11.9*	1.18 ± 0.02*
LMWH and PGE_1	ND	125.8 ± 33.6*	ND
LMWH Hct 60	ND	ND	1.16 ± 0.02*
Citrate and LMWH	30.8 ± 2.5	166.5 ± 25.6†	$87.3 \pm 11 \times 10^{-3}$†

Values are mean ± SEM.
*Value differs significantly ($P < 0.05$) from corresponding value for samples containing only unadjusted concentrations of sodium citrate. †Value differs significantly ($P < 0.05$) from corresponding value for samples containing only unadjusted concentrations of LMWH.
ND = Not done.

> 50 µ*M* $CaCl_2$ and slightly curvilinear for values less than that. Coefficients of variation in the linear and curvilinear ranges of the curve were 1 and 1.4%, respectively.

Statistical analysis—Results were analyzed by use of an ANOVA for repeated measures[m] to examine the effect of anticoagulant and PGE_1. When the F statistic was significant, appropriate comparisons were made, using the Bonferroni post-hoc test. Differences were considered significant for $P < 0.05$. All results were reported as mean ± SEM.

Results

Hematocrit and platelet count—The Hct did not differ significantly in samples collected into syringes containing the various anticoagulants (**Table 1**). The Hct for the 4 horses ranged from 25 to 38% (mean ± SEM, 31.5 ± 3%).

Platelet counts in samples containing LMWH (109.5 ± 11.2 X 10^3 cells/µl) were significantly less than counts in samples containing sodium citrate (187.3 ± 30.3 X 10^3 cells/µl; Table 1). Platelet count in samples containing LMWH and sodium citrate was intermediate (166.5 ± 25.6 X 10^3 cells/µl) between samples containing LMWH or sodium citrate and was significantly greater than values for samples containing LMWH. Platelet count in samples collected into syringes containing sodium citrate and PGE_1 (187.5 ± 25.9 X 10^3 cells/µl) was not significantly different from the count in samples containing sodium citrate. The addition of PGE_1 to LMWH did not significantly alter the platelet count (125.7 ± 33.6 X 10^3 cells/µl), compared with samples containing LMWH, and was significantly less than the count in samples containing sodium citrate.

Microscopic evaluation of smears of PRP and whole blood indicated there was clumping of platelets in samples collected into syringes containing LMWH that was not evident in samples containing sodium citrate. There was some platelet clumping observed in samples collected into syringes containing a combination of LMWH and sodium citrate, although it was less than that observed in the samples containing only LMWH. This finding was consistent among horses. We did not attempt to semiquantitatively evaluate the degree of clumping.

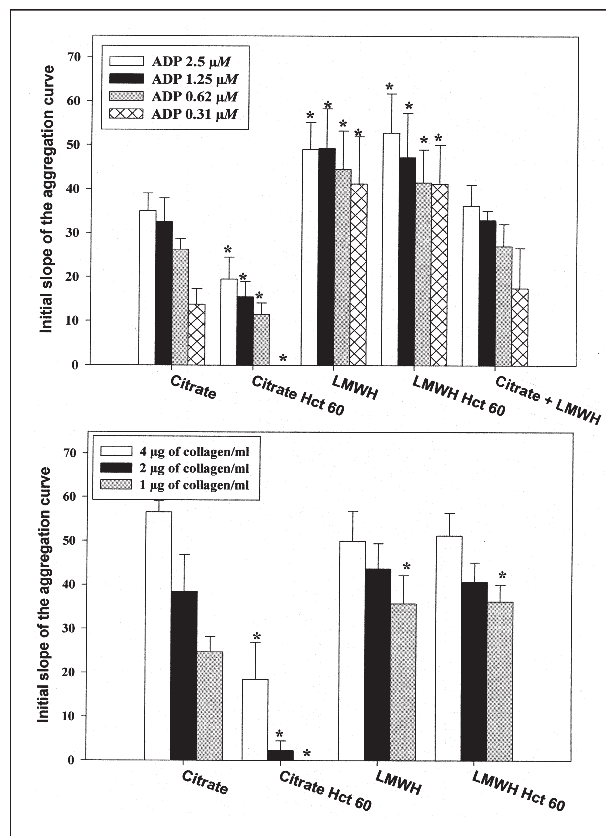

Figure 1—Mean ± SEM aggregation responses of platelets to 2.5, 1.25, 0.625, and 0.3125 µ*M* ADP (top) or 4, 2, and 1 µg of collagen/ml of blood (bottom) in blood samples collected into syringes containing sodium citrate (citrate), sodium citrate adjusted to the calculated concentration in a sample with a hematocrit of 60% (citrate Hct 60), low molecular weight heparin (LMWH), LMWH adjusted to the calculated concentration in a sample with a hematocrit of 60% (LMWH Hct 60), and a combination of sodium citrate and LMWH. *Value differs significantly ($P < 0.05$) from corresponding value for samples containing only an unadjusted concentration of sodium citrate.

Platelet aggregation—Platelet aggregation responses were significantly greater for all concentrations of ADP in samples containing LMWH, compared with samples containing sodium citrate (**Fig 1**). At the

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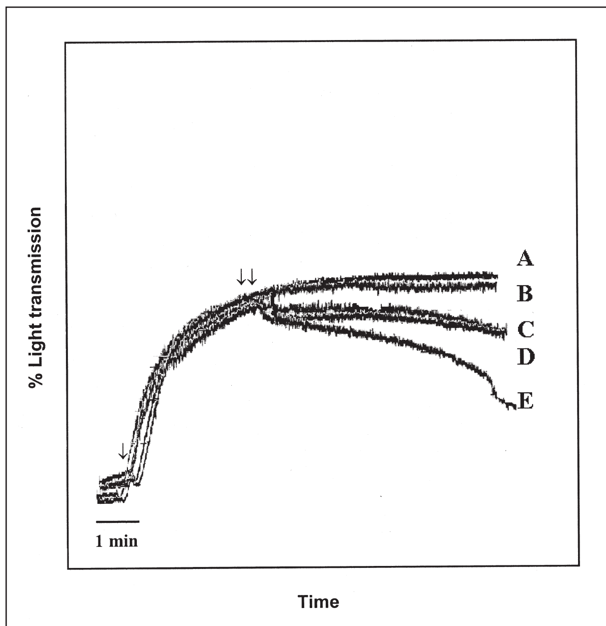

Figure 2—Representative tracings of the aggregation response in samples (obtained from 1 horse) after addition of 2.5 µ*M* ADP. Line A = Sample containing LMWH, typical aggregation response to ADP. Line B = Saline (0.9% NaCl) solution added 4 minutes after ADP. Line C = Sodium citrate (calculated concentration for a sample with a Hct of 35%) added 4 minutes after ADP. Line D = Sodium citrate (calculated concentration for a sample with a Hct of 50%) added 4 minutes after ADP. Line E = Sodium citrate (calculated concentration for a sample with a Hct of 60%) added 4 minutes after ADP. ↓ = Addition of ADP. ↓↓ = Addition of saline solution or sodium citrate.

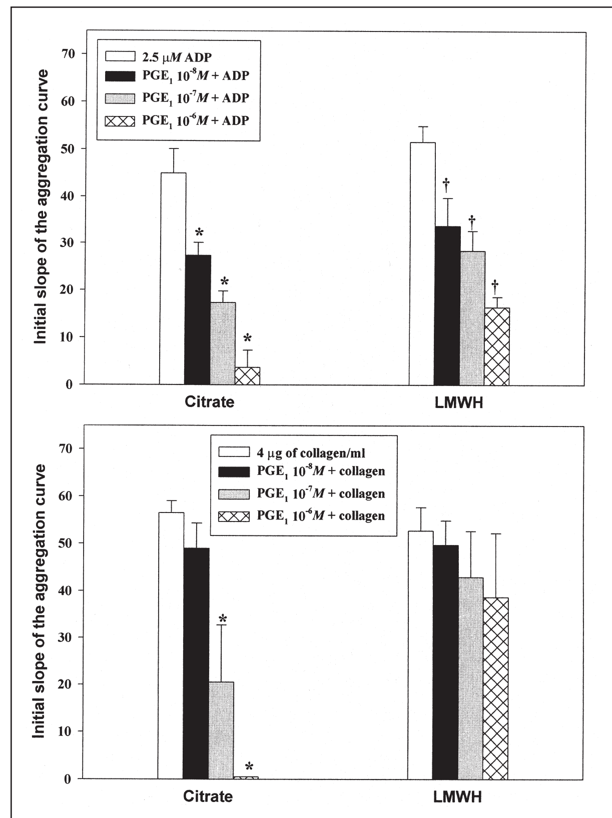

Figure 3—Mean ± SEM aggregation responses of platelets to 2.5 µ*M* ADP (top) or 4 µg of collagen/ml (bottom) after the addition of various concentrations of prostaglandin E_1 (PGE_1) to blood samples were collected into syringes containing sodium citrate or LMWH. *Value differs significantly ($P < 0.05$) from value for samples containing sodium citrate that did not contain any PGE_1. †Value differs significantly ($P < 0.05$) from value for samples containing LMWH that did not contain any PGE_1.

lower concentrations of collagen, responses in samples containing LMWH were greater than those containing sodium citrate as the anticoagulant. The aggregation responses to ADP in samples containing a combination of LMWH and sodium citrate were similar to those observed in samples containing only sodium citrate. Responses to collagen were not evaluated in samples containing a combination of LMWH and sodium citrate. Increasing concentrations of sodium citrate in samples resulted in significant reductions in platelet aggregation responses, whereas there was not a detectable effect for increasing concentrations of LMWH. When sodium citrate was added 4 minutes after addition of 2.5 µ*M* ADP, platelet aggregation was partially reversed in a concentration-dependent manner (**Fig 2**). The addition of sodium citrate to samples activated with collagen did not result in reversal of aggregation (results not shown).

Addition of PGE_1 to PRP before addition of ADP or collagen resulted in inhibition of platelet aggregation responses in a concentration-dependent manner (**Fig 3**). When PGE_1 was added 4 minutes after 2.5 µ*M* ADP, aggregation was partially reversed in a concentration-dependent manner (**Fig 4**). However, when $10^{-6}M$ PGE_1 was added 4 minutes after addition of 4 µg of collagen/ml, aggregation was not reversed (results not shown).

Platelet activation—Total fluorescence of unactivated platelets did not differ significantly in samples collected into syringes containing LMWH or sodium citrate or after the addition of PGE_1 (**Table 2**). Fluorescence increased significantly in ADP-stimulated samples collected into syringes containing sodium citrate, LMWH, and a combination of LMWH and sodium citrate. A similar increase in fluorescence was observed in collagen-activated samples. Addition of PGE_1 to ADP-activated samples resulted in a concentration-dependent reduction in total fluorescence in response to the 2 highest concentrations of PGE_1 (**Fig 5**). Total fluorescence in ADP-stimulated samples decreased from 791 ± 143.9 arbitrary fluorescence units in the original sample to 639 ± 17.9, 249 ± 14.3, and 173 ± 31.6 arbitrary fluorescence units after the addition of sodium citrate to achieve a final concentration equivalent to that in samples with a Hct of 35, 50, and 60%, respectively. However, a reduction in fluorescence was not observed in collagen-activated samples after the addition of PGE_1 or sodium citrate (results not shown). Total fluorescence in unactivated platelets was significantly greater than in the negative-control sample.

Plasma concentration of ionized calcium—Ionized calcium concentration in samples containing LMWH (1.18 ± 0.02 m*M*) was significantly higher than the concentration in samples containing sodium citrate

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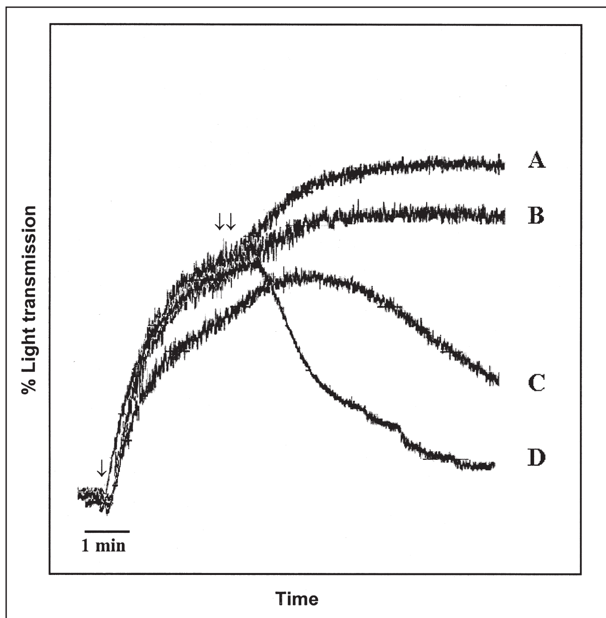

Figure 4—Representative tracings of the aggregation response for blood samples (obtained from 1 horse) after addition of 2.5 μM ADP. Line A = Sample containing sodium citrate, typical aggregation response to ADP. Line B = $10^{-8}M$ PGE_1 added 4 minutes after ADP. Line C = $10^{-7}M$ PGE_1 added 4 minutes after ADP. Line D = $10^{-6}M$ PGE_1 added 4 minutes after ADP. ↓ = Addition of ADP. ↓↓= Addition of PGE_1.

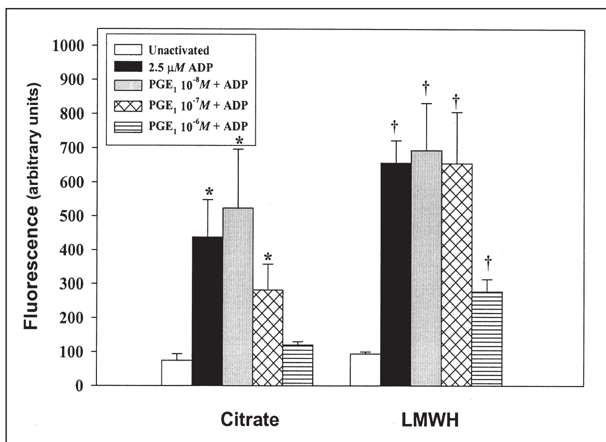

Figure 5—Mean ± SEM total fluorescence, determined by use of flow cytometry, of unactivated platelets, platelets activated with 2.5 μM ADP, and activated platelets exposed to various concentrations of PGE_1 for blood samples collected into syringes containing sodium citrate or LMWH. *Value differs significantly ($P < 0.05$) from value for unactivated platelets in samples containing sodium citrate. †Value differs significantly ($P < 0.05$) from value for unactivated platelets in samples containing LMWH.

($75 \pm 5.6 \times 10^{-3}$ m*M*; Table 1). When sodium citrate was adjusted to a concentration equivalent to that in a sample with a Hct of 60%, there was a significant decrease in plasma concentration of ionized calcium ($30.4 \pm 4.6 \times 10^{-3}$ m*M*). Increasing the amount of LMWH in samples did not significantly affect ionized calcium concentration (1.16 ± 0.01 m*M*). Plasma concentration of ionized calcium in samples containing a combination of LMWH and sodium citrate was $87.3 \pm 11 \times 10^{-3}$ m*M*.

Table 2—Total fluorescence, determined by use of flow cytometry, of negative-control platelets (collected into syringe containing sodium citrate and incubated with fluorescein-conjugated goat anti-mouse IgG), unactivated platelets, and platelets stimulated with 2.5 μM ADP or 4 μg of collagen/ml

Sample	Fluorescence
Negative control	6.9 ± 1.1*
Citrate	74.5 ± 19.6
Citrate and PGE_1	74.7 ± 14.7
Citrate Hct 60	62.1 ± 11.8
Citrate and ADP	437.8 ± 110.5*
Citrate and collagen	337.2 ± 38.9*
LMWH	95.8 ± 5.4
LMWH and PGE_1	93.9 ± 6.3
LMWH Hct 60	86.1 ± 6.3
LMWH and ADP	659.9 ± 65†
LMWH and collagen	590.5 ± 58.5†
Citrate and LMWH	64.3 ± 13.2
Citrate and LMWH and ADP	548.9 ± 128.7*†

Values are mean ± SEM.

*Value differs significantly ($P < 0.05$) from corresponding value for samples containing only sodium citrate without any agonist. †Value differs significantly ($P < 0.05$) from corresponding value for samples containing only LMWH without any agonist. *See* Table 1 for remainder of key.

Discussion

Analysis of the findings of the study reported here indicates that the anticoagulant used in a study should be considered when interpreting results for tests assessing the function of equine platelets. A novel finding was a significantly lower platelet count in samples collected into syringes containing LMWH, compared with counts for samples collected into syringes containing sodium citrate. Evaluation of smears of samples indicated a tendency of platelets to clump when collected into syringes containing LMWH. This clumping was reduced when samples were collected into syringes containing a combination of LMWH and sodium citrate. It is unclear whether clumping was the result of direct activation of platelets by LMWH or some other mechanism. Although the aggregation response was greater in samples containing LMWH, compared with that for samples containing sodium citrate, this greater response was not seen in samples containing a combination of sodium citrate and LMWH. Similarly, we did not detect a difference between the amount of fibrinogen binding to platelets in samples containing sodium citrate, compared with values for samples containing a combination of LMWH and sodium citrate. It is possible that the higher concentration of ionized calcium in samples containing LMWH increased the likelihood of in vitro activation of platelets and, therefore, platelet clumping. In our experience, we believe that samples collected into syringes containing lesser amounts of sodium citrate also have a greater tendency to clump. This observation adds support to the idea that total calcium concentration in PRP samples may impact the degree of in vitro activation of platelets.

Unfractionated heparins have a proaggregatory effect on platelets, resulting in platelet clumping and aggregation.[12,13] People and horses treated by systemic administration of unfractionated heparins can develop thrombocytopenia.[26-29] The heparin molecule has 2 binding sites. One is capable of binding to antithrombin III and platelets, and the other binds to platelets.[30] Binding of unfractionated heparins to platelets con-

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tributes to platelet clumping and thrombocytopenia. The preferential binding site of heparin to antithrombin III is confined to low molecular weight fractions of heparin. Hence, LMWH has been associated with less platelet activation and thrombocytopenia.[13,31] Nonetheless, thrombocytopenia has been reported in people treated with LMWH.[32] It is believed that thrombocytopenia observed with use of LMWH is an immune-mediated problem.[33] There is limited information about the use of LMWH in horses, but investigators did not detect evidence that treatment with LMWH resulted in thrombocytopenia in 1 study.[29] Whereas there was evidence of platelet clumping in the study reported here, LMWH did not appear to increase platelet activation as measured by degree of fibrinogen binding. However, background binding of fibrinogen to fixed equine platelets was substantial (Table 2) and may have masked subtle differences in platelet activation between platelets collected into syringes containing sodium citrate instead of LMWH. Fixation of platelets with formaldehyde increases the surface expression of activation markers[34] and likely caused the increased binding of fibrinogen observed in our study. Therefore, we cannot be certain that samples containing LMWH caused some activation of platelets. Although the amplitude and slope of aggregation curves were greater for samples containing LMWH, this does not necessarily indicate that we were assessing normal platelet aggregation. The clumping of platelets secondary to the addition of LMWH may have artificially affected platelet aggregation under these conditions. Therefore, given the tendency of equine platelets to clump in samples containing LMWH, it cannot be recommended as a suitable anticoagulant for assessing responses of equine platelets.

Results from the study reported here documented that platelet aggregation was reduced when plasma concentration of sodium citrate was increased in samples. In those samples, there was a significantly lower concentration of ionized calcium. However, the reported values for plasma concentration of ionized calcium in our study are significantly lower than has been described elsewhere.[35] This was likely attributable to the effect of freezing and storage of the samples.[36] Nonetheless, although the values reported here are lower than may be expected, all samples were stored and handled in the same manner. Therefore, the results should provide a good indication of the differences between the anticoagulants.

Addition of sodium citrate to a sample during aggregation with ADP caused partial reversal of the aggregation process. This finding reflects the importance of extracellular calcium for platelet function. Changes in extracellular calcium concentration directly affect the results for tests of platelet function. Bell et al[15] reported that differences in platelet responses between samples obtained from males and females were attributable to variations in extracellular concentrations of calcium. By adjusting the extracellular concentration of calcium, they were able to correct the difference in platelet responses attributable to sex of the subject. The differing extracellular concentration of calcium in males and females was attributable to a vast difference in Hct. Such a difference in Hct directly affects plasma concentration of sodium citrate, which in turn affects plasma concentration of ionized calcium.

Sodium citrate acts by chelating calcium. When sodium citrate is added to blood samples, it is not taken up by the cells.[15] Hence, plasma concentration of a given amount of sodium citrate and, therefore, concentration of extracellular calcium in a blood sample are dependent on the Hct of that sample. Horses develop polycythemia associated with exercise, with Hct often increasing from 40 to 60% or greater. When blood is collected in the traditional proportion of 9 parts blood:1 part sodium citrate (0.11*M* or 3.2%), then samples with a Hct of 60% will contain 150% of the sodium citrate in the plasma component of the blood sample, compared with samples with a Hct of 40%. The resulting reduction in ionized calcium concentration would lead to reduced responsiveness of platelets in samples collected during or immediately after exercise. Hence, reduced aggregation described by Bayly et al[5] and Johnstone et al[7] likely was attributable, at least in part, to the result of reduced availability of calcium. Given the tendency of platelets to clump in samples containing LMWH, the increased aggregation in samples collected during exercise may be artifactual.[4] However, it also is possible that an increase in plasma concentration of calcium associated with maximal exercise of horses[37] may be a contributing factor. Therefore, before further conclusions can be reached regarding the effects of exercise on function of equine platelets, alterations in plasma concentration of calcium need to be considered.

Prostaglandin E_1 is a metabolite of arachidonic acid that often is used to prevent in vitro activation of platelets.[11,20] Inhibitory actions of PGE_1 are the result of its ability to increase cytoplasmic cAMP, which leads to reduced mobilization of calcium ions from internal stores or from the external environment.[38] When PGE_1 is added prior to addition of an agonist, platelet activation, aggregation, and binding of fibrinogen are inhibited.[23,38-40] It also has been reported that PGE_1 may cause reversal of aggregation[23,41] and fibrinogen binding[22,23,41] when added to activated samples. We were able to document a concentration-dependent effect of PGE_1 on aggregation and fibrinogen binding of equine platelets in samples activated with ADP. Such an effect raises concerns about the use of PGE_1 in samples intended for assessment of platelet activation. Addition of PGE_1 may reduce fi brinogen binding in activated samples and result in an underestimation of the degree of platelet activation. The inability of Weiss et al[3] to detect platelet activation during exercise by measurement of fibrinogen binding may have been partially attributable to the use of PGE_1. Nonetheless, there also is evidence that the use of PGE_1 does not interfere with fibrinogen binding for detection of activation of equine platelets. Russell et al[2] reported increased fibrinogen binding in activated platelets from ponies with equine infectious anemia virus in samples collected into tubes containing PGE_1. It is probable that the extent of fibrinogen binding and its reversibility after addition of PGE_1 are dependent on the method by which

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platelets are activated and the duration of activation. Studies of human and rabbit platelets revealed that platelets were less likely to deaggregate when exposed to PGE_1 if they had undergone the release reaction (serotonin secretion) induced by collagen or thrombin and that this tendency was greater in human platelets.[42] Indeed, our results revealed that equine platelets activated with 4 µg of collagen/ml did not deaggregate or lead to reduced fibrinogen binding when exposed to PGE_1. Other investigators have reported that 100 µ*M* ADP causes release of only 4% or less of total serotonin from equine platelets, whereas 3 µg of collagen/ml results in release of 35%.[43] Therefore, it seems probable that use of PGE_1 to reverse aggregation of equine platelets is dependent on whether the platelets have undergone secretion. Because the mechanism of platelet activation is generally undefined for in vivo studies, investigators should be careful when interpreting negative findings in samples containing PGE_1. Depending on the mechanism of platelet activation, PGE_1 may reverse fibrinogen binding and impede the ability to detect activated platelets.

Investigators must be careful when interpreting results of tests evaluating responses of equine platelets and must consider the anticoagulant used. Furthermore, variations in plasma concentrations of sodium citrate secondary to differences in Hct have the potential to lead to substantial differences in extracellular concentration of calcium. Hence, altered responses of platelets may be associated with the ionized calcium concentration rather than other factors such as exercise. When evaluating platelets in samples in which there are large differences in Hct, investigators should ensure that plasma concentrations of sodium citrate are similar in all samples to avoid anticoagulant-induced variations in results. Although LMWH does not have a significant effect on extracellular concentration of calcium, it does not appear to be a good anticoagulant for assessment of responses of equine platelets because of the tendency of platelets to clump in samples containing LMWH.

[a]Sodium citrate, JT Baker Inc, Phillipsburg, NJ.
[b]Fragmin, Pharmacia & Upjohn Co, Kalamazoo, Mich.
[c]Prostaglandin E_1, Sigma Chemical Co, St Louis, Mo.
[d]Baker 9010 plus, Bio Immunochem, Allentown, Pa.
[e]Whole-blood aggregometer, Chrono-log Corp, Havertown, Pa.
[f]Agglink, Chrono-log Corp, Havertown, Pa.
[g]ADP Grade I, Chrono-log Corp, Havertown, Pa.
[h]Collagen reagent, Chrono-log Corp, Havertown, Pa.
[i]Fibrinogen polyclonal antibody, Cappel Research Products, ICN Biomedicals Inc, Costa Mesa, Calif.
[j]IgG $F(ab')_2$ polyclonal antibody, ICN Biomedicals Inc, Costa Mesa, Calif.
[k]FACScan, Becton-Dickinson, San Jose, Calif.
[l]Model 97-20, ORION, Beverly, Mass.
[m]NCSS 2000, Number Cruncher Statistical Systems, Kaysville, Utah.

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