Effects of formaldehyde fixation on equine platelets using flow cytometric methods to evaluate markers of platelet activation

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Objective—To investigate the effects of formaldehyde fixation on equine platelets using flow cytometric methods to evaluate markers of platelet activation.

Sample Population—Blood samples from 6 Thoroughbreds.

Procedure—The degree of fluorescence associated with binding of fluorescein isothiocyanate (FITC)-conjugated anti-human fibrinogen antibody and FITC-annexin V in unactivated and adenosine diphosphate (ADP)-, platelet activating factor (PAF)-, and A23187-activated platelet samples in unfixed and 0.5, 1.0, and 2.0% formaldehyde-fixed samples was assessed by use of flow cytometry.

Results—In samples incubated with FITC-anti-human fibrinogen antibody prior to fixation, addition of 2.0% formaldehyde resulted in a 30% increase in total fluorescence in ADP- and PAF-activated samples and a 60% increase in A23187-activated samples. Fixation for 24 hours prior to addition of antibody resulted in reduced fluorescence of samples containing anti-human fibrinogen antibody for all 3 concentrations of formaldehyde in PAF-activated samples. The addition of all 3 concentrations of formaldehyde after incubation with FITC-annexin V resulted in significant increases in fluorescence in unactivated and activated platelet samples. As length of fixation time increased, there was a gradual increase in fluorescence that was significant at 24 hours.

Conclusions—Because fixation with 2.0% formaldehyde results in significant changes in fluorescence in activated platelet samples containing anti-fibrinogen antibody, lower concentrations of formaldehyde should be used to fix equine platelet samples. Formaldehyde-fixed platelet samples should be analyzed within 12 hours of fixation to avoid artifactual increases in fluorescence. Fixation of samples containing FITC-annexin V should be avoided because of significant increases in fluorescence that may interfere with interpretation of results. (*Am J Vet Res* 2002;63:840–844)

Received Jul 16, 2001.
Accepted Jan 8, 2002.
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Supported by the T. R. Pelley Memorial Fund, the Washington State Equine Research Program, and the Robert B. McEachern Endowment.
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Flow cytometry has become a widely accepted tool for assessing in vivo and in vitro platelet activation.[1,2] The method has the advantage of minimizing in vitro platelet activation by rapidly analyzing samples and eliminating the need to centrifuge and wash the samples. However, problems may arise when there is a delay in sample preparation time or availability of a flow cytometer. Fixation of cells has been used to increase the ease of analysis and extend the time in which a useful analysis may be performed. The preferred fixative for platelets is fresh formaldehyde generated from paraformaldehyde powder and is often referred to as paraformaldehyde. This is favored over commercially available formaldehyde (formalin), as the latter contains impurities such as methanol and formic acid.[3] Optimal methods for use of fixatives have not been established. In some instances platelet samples are fixed prior to incubation with fluorescent-labeled molecular markers, and in other instances fixation is employed following incubation. In addition, there is some variation in concentration of fixative employed. Ideally, the chosen fixative solution should not cause artifactual changes in results. Although the results of several studies[4,5] indicate fixation does not alter expression of activation markers, others have demonstrated fixative-induced increases in platelet P-selectin expression,[6] fibrinogen binding,[7] and formation of platelet-leukocyte aggregates.[8] However, Hu et al[9] reported that problems with fixation could be avoided with 0.5 to 1.0% solutions of formaldehyde.

There are a limited number of methods for assessing equine platelet activation because of poor species cross-reactivity of many of the molecular markers.[10] Available techniques include assessment of platelet fibrinogen binding by use of an anti-human fibrinogen antibody[11,12] and phosphatidylserine expression detected via binding of annexin V.[13] These methods have been described with and without the use of fixation. There is evidence that fixation alters binding of fibrinogen to human platelets[7]; however, it is unknown if similar changes are evident by use of equine samples. Therefore, this study was undertaken to evaluate the impact of fixation and formaldehyde concentration on measures of equine platelet activation. It was also intended to evaluate sample stability of formaldehyde-fixed samples over time.

Materials and Methods

Animals—Six healthy sedentary Thoroughbreds (6 to 17 years of age) were used in the study. All procedures were conducted in compliance with guidelines established by the Washington State University Animal Care and Use Committee.

Sample preparation—Ten milliliters of venous blood was collected via venipuncture of the left jugular vein by use

of 18-gauge needles and syringes containing one-tenth volume of 0.11*M* sodium citrate.[a] Following venipuncture, the samples were transferred to sterile plastic tubes, and erythrocytes were allowed to settle for 20 minutes, then samples were processed immediately. Blood was collected from each horse on 2 separate occasions, once for the experiments that involved fixation after the addition of molecular marker and once for experiments where samples were fixed for 24 hours before addition of molecular marker.

Samples formaldehyde fixed after incubation with molecular marker—Ten microliters of the **platelet and leukocyte-rich plasma (PLRP)** layer was added to tubes containing 250 µl of 0.5% **bovine serum albumin (BSA)** in **HEPES-buffered Tyrodes solution (HBTS**: 145 m*M* NaCl, 2.7 m*M* KCl, 0.42 m*M* $NaHPO_4$, 12 m*M* $NaHCO_3$, 5.5 m*M* dextrose, and 5 m*M* HEPES; pH 7.4) and saturating concentrations of diluted **fluorescein isothiocyanate (FITC)**-conjugated goat $F(Ab')_2$ fragment to human fibrinogen antibody[b] (1:250) or FITC-conjugated goat $F(Ab')_2$ fragment to mouse IgG (1:250; control antibody[c]). Alternatively, PLRP was diluted 1 to 5 in **phosphate buffered saline (PBS)** solution, after which 20 µl of diluted PLRP was added to 250 µl of annexin binding buffer[d] (140 m*M* NaCl, 2.5 m*M* $CaCl_2$, and 10 m*M* HEPES and NaOH; pH 7.4) and 5 µl of FITC-annexin V.[e] The appropriate saturating concentrations of antibodies and FITC-annexin V and dilution of PLRP had been determined in preliminary studies. In activated samples, **adenosine diphosphate[f] (ADP)**, **platelet activating factor[g] (PAF)**, or A23187[h] were also added at the final concentrations of 10 or 0.625 µ*M*, 10 or 0.1 n*M*, or 10 or 2.5 µ*M*, respectively. After gentle mixing, samples were incubated at room temperature (20 to 22 C) in the dark for 20 minutes. Afterwards, the incubation was terminated by the addition of 750 µl of 0.5% BSA in HBTS or PBS solution containing freshly prepared formaldehyde to give final concentrations of 0.5, 1, or 2% formaldehyde. For assessment of stability of the formaldehyde-fixed samples, samples were analyzed immediately after preparation (time 0) and after storage in the dark for durations of 2, 6, 12, and 24 hours.

Samples formaldehyde-fixed prior to incubation with molecular marker—Ten microliters of PLRP was added to 250 µl of 0.5% BSA in HBTS as described and stored in 0.5, 1, or 2% formaldehyde for 24 hours at 4 C. In addition, platelets were activated with 10 n*M* and 0.1 n*M* PAF prior to fixation. Following overnight incubation, formaldehyde-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% BSA in HBTS. Platelets then were washed once in 0.5% BSA in HBTS and centrifuged at 1,000 × g for 2 minutes. The pellet was resuspended in 250 µl of 0.5% BSA in HBTS containing FITC-conjugated goat $F(Ab')_2$ fragment to human fibrinogen antibody at a dilution of 1:250. Samples were incubated for 20 minutes in the dark then diluted with 750 µl of HBTS and analyzed immediately.

Flow cytometric analysis—Samples were analyzed with a flow cytometer.[i] All variables were collected by use of 4-decade logarithmic amplification. The platelet population was identified by forward- and side-scatter characteristics, and 10,000 gated events were measured for each sample as described previously.[13] The gated events were then subjected to single-color (FITC-annexin V or FITC-anti-fibrinogen antibody) analyses to observe the geometric mean of the total fluorescence intensity of the platelet population, which included background fluorescence.

Statistical analysis—Results were analyzed by use of an ANOVA for repeated measures[j] to examine the effect of fixative and time. When the F statistic was significant, appropriate comparisons were made by use of the Bonferroni post hoc test. Differences were considered significant for values of $P < 0.05$. All results were reported as the mean ± SEM of total fluorescence in **arbitrary units (AU)**.

Results

Effects of formaldehyde on platelet fibrinogen binding measurement—Without in vitro activation, the total fluorescence of unfixed (0.5% BSA in HBTS diluted) samples was 53.5 ± 3.8 AU. This was not significantly different from 2.0 (61.1 ± 4.3 AU), 1.0 (52.3 ± 4.3 AU), or 0.5% (51.6 ± 3.4 AU) formaldehyde-fixed samples. Results were similar for the control antibody (results not shown). Activation with 10 µ*M* ADP, 10 n*M* PAF, and 10 µ*M* A23187 resulted in increased fluorescence in anti-fibrinogen antibody samples, and this fluorescence was further increased significantly ($P = 0.02$) in samples fixed in 2.0% formaldehyde (**Fig 1**). Similar findings were seen at lower agonist concentrations (data not shown).

In the samples fixed 24 hours prior to incubation

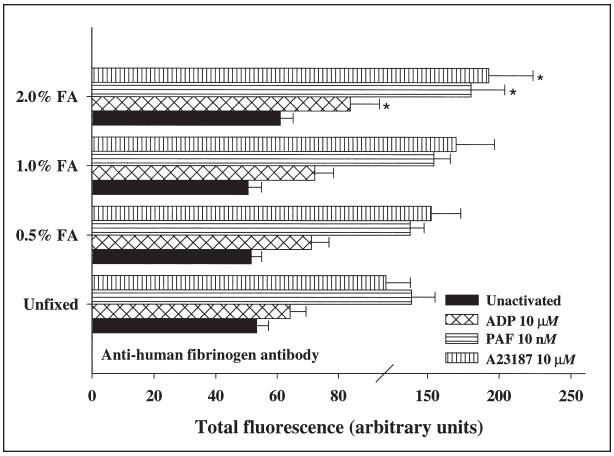

Figure 1—Mean (± SEM) total fluorescence, determined by use of flow cytometry, of unactivated platelets and platelets activated with adenosine diphosphate (ADP), platelet activating factor (PAF), or A23187, in buffer (unfixed) or 0.5, 1.0, or 2.0% formaldehyde (FA) added after incubation with fluorescein-labeled anti-human fibrinogen antibody. *Value differs significantly ($P < 0.05$) from value for unfixed platelet samples

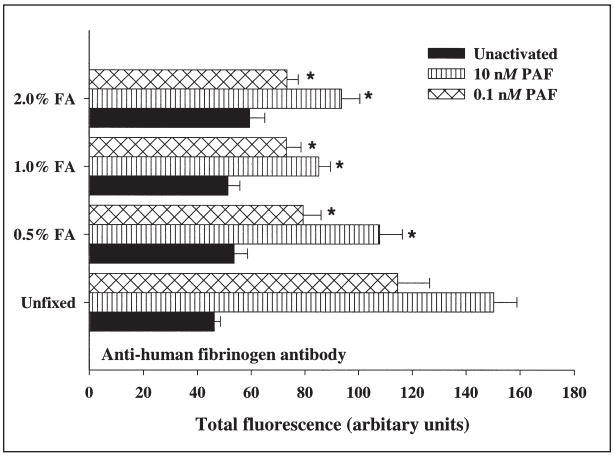

Figure 2—Mean (± SEM) total fluorescence of unactivated platelets and platelets activated with PAF, in buffer (unfixed) or incubated for 24 hours in 0.5, 1.0, or 2.0% FA prior to addition of fluorescein-labeled anti-human fibrinogen antibody. *See* Figure 1 for key.

with the anti-human fibrinogen antibody, the total fluorescence in unactivated platelet samples was not significantly (P = 0.18) different from the unfixed samples. However, fluorescence was significantly (P = 0.001) reduced in 10 n*M* PAF-activated samples (**Fig 2**) and in 0.1 n*M* PAF-activated samples (data not shown) in all 3 concentrations of formaldehyde.

Effects of formaldehyde on platelet annexin V binding measurement—Without in vitro activation, platelets incubated with annexin V-FITC had a significant (P = 0.008) increase in fluorescence for all concentrations of formaldehyde, compared with unfixed samples. There was also a significant (P = 0.01) increase in fluorescence of samples activated with both concentrations of PAF and A23187 but not with ADP in formaldehyde-fixed versus unfixed samples (**Fig 3**).

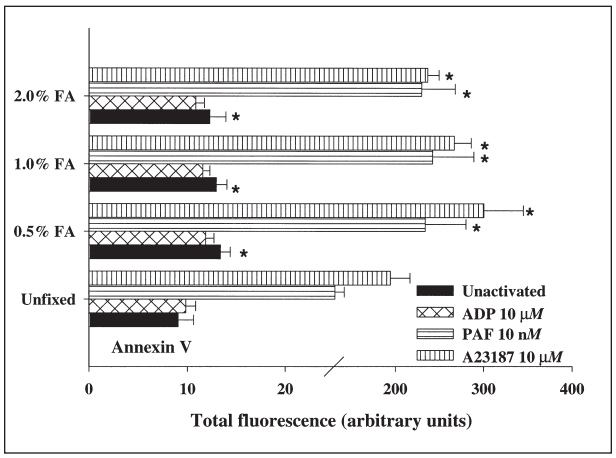

Figure 3—Mean (± SEM) total fluorescence of unactivated platelets and platelets activated with ADP, PAF, or A23187, in buffer (unfixed) or 0.5, 1.0, or 2.0% FA added after incubation with fluorescein-labeled annexin V. *See* Figure 1 for key.

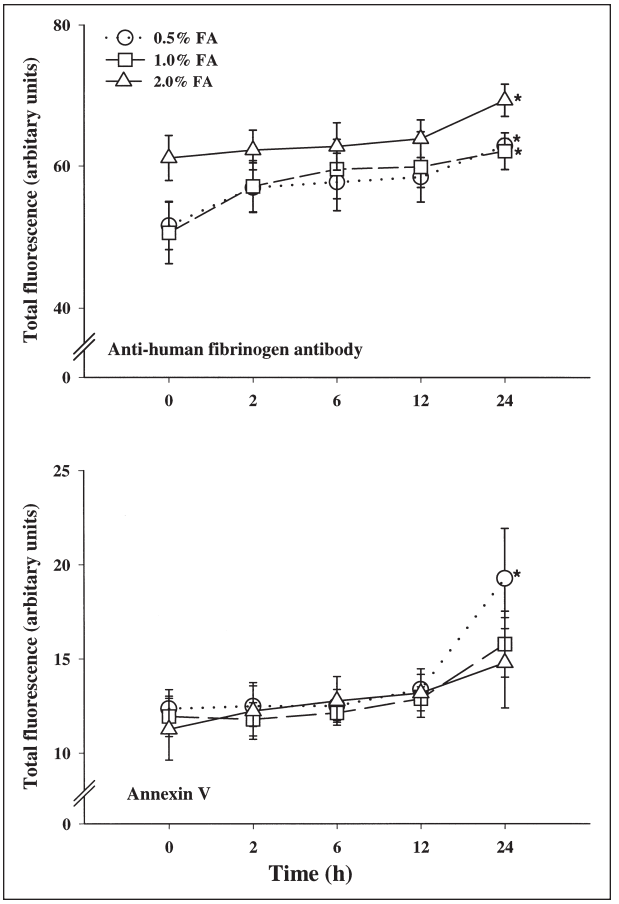

Figure 4—Stability of unactivated platelet samples fixed in FA that was added after incubation with fluorescein-labeled anti-human fibrinogen antibody (top) or annexin V (bottom), immediately after addition of FA (time 0) and at 2, 6, 12, and 24 hours. *Value significantly ($P < 0.05$) different from corresponding value at time 0.

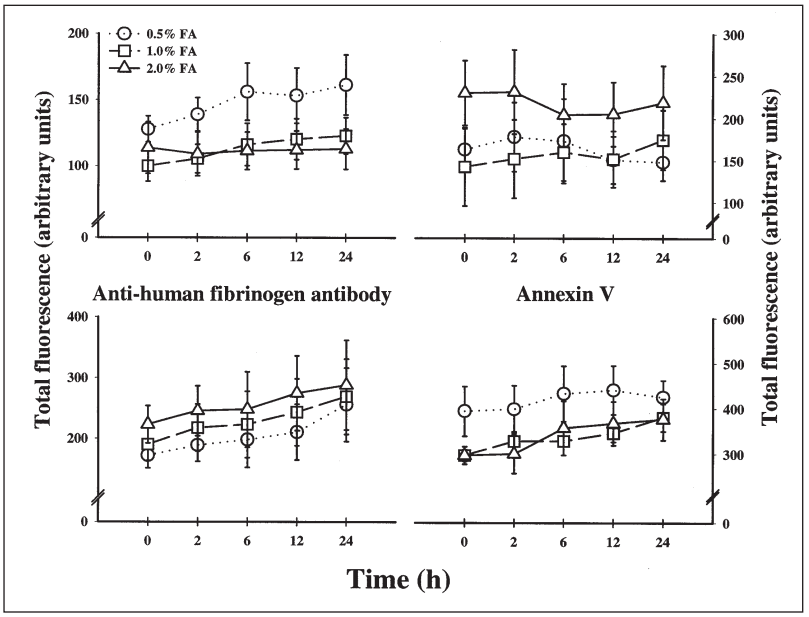

Figure 5—Stability of 10 n*M* PAF (top) and 10 μ*M* A23817-activated (bottom) platelet samples fixed in FA that was added after incubation with fluorescein-labeled anti-human fibrinogen antibody (left) or annexin V (right), immediately after addition of FA (time 0) and at 2, 6, 12, and 24 hours.

Stability of formaldehyde-fixed samples— Although there was a gradual increase in fluorescence over time, this was not significant in the anti-fibrinogen antibody samples until 24 hours ($P = 0.03$) for all 3 concentrations of formaldehyde (**Fig 4**). For annexin V-FITC samples, only the 0.5% formaldehyde had a significant ($P = 0.009$) increase at 24 hours.

In ADP-activated samples (10 and 0.625 μ*M*), there was a gradual increase in fluorescence over time, which was significant ($P = 0.03$) at 24 hours, similar to that seen in the unactivated samples (data not shown). However, 10 n*M* PAF- and 10 μ*M* A23187-activated samples (**Fig 5**) and 0.1 n*M* PAF- and 2.5 μ*M* A23187-activated samples (data not shown) had no significant ($P = 0.12$) change over time.

Discussion

The results of our study indicate that fixation of equine platelet samples with formaldehyde has the potential to significantly alter fluorescence associated with binding of anti-fibrinogen antibody and annexin V. The effects of fixation were evaluated in unactivated and activated platelet samples. Three agonists with various mechanisms of action and ability to activate platelets were selected. These were chosen to evaluate the potential effect of fixation on platelets at varying degrees of activation. In addition, binding of annexin V to platelets is dependent on the surface expression of phosphatidylserine.[14] Only strong agonists such as PAF and the calcium ionophore A23187 cause surface expression of phosphatidylserine and secretion in equine platelets,[13] whereas ADP is a weak agonist that drives equine platelet aggregation but not secretion[15,16] or surface expression of phosphatidylserine.[13] With regards to anti-fibrinogen antibody, the greatest effect was evident when using 2.0% formaldehyde. Although a significant effect was not evident in unactivated platelet samples, activated samples had greater fluorescence intensity of anti-fibrinogen antibody samples when 2.0% formaldehyde was added after incubation with antibody. A similar finding has been reported with human platelets using 0.2% formaldehyde.[7] In contrast, in samples fixed prior to addition of antibody, the degree of fluorescence was significantly reduced in activated samples at all concentrations of formaldehyde. Aldehyde fixatives have the potential to create cross-links between amino acid residues or proteins and can denature proteins.[3] Therefore, formaldehyde could create cross-links between antibodies and platelet membrane proteins or between bound and free antibody, which could increase nonspecific antibody binding to platelets.[9] Because platelet samples are not washed to remove excess antibody, there is probably an increased likelihood of cross-links developing. However, although several studies[6,7] have reported increased binding of antibodies in formaldehyde-fixed samples, others report no change or even decreased binding.[4,5,9] Such disparity in findings probably relates to dissimilar experimental conditions, as well as the antibodies, buffers, and fixative concentrations used. Therefore, it is important for each research laboratory to carefully evaluate fixation methods before designing protocols for platelet assays using flow cytometry.

Fixation of samples prior to incubation with antibody has the advantage of extending the time between sample collection and analysis. It also allows for complete removal of fixative before addition of antibody, through several washing steps. We chose to leave samples in fixative for 24 hours, as this method has been previously described in horses[12] and could be a more convenient method in many research or clinical situations. Although we were unable to show a significant fixative effect in unactivated platelet samples, fixation resulted in reduced fluorescence in PAF-activated samples. Given that fixatives can denature proteins,[3] it is possible that overnight fixation of samples resulted in denaturing of bound fibrinogen and a resultant loss of bound fibrinogen. Alternatively, there may have been changes in fibrinogen structure rendering it less recognizable to the antibody. We are unsure how this might impact samples containing platelets activated in vivo. Although we were still able to clearly demonstrate activation of platelet samples that had been maximally stimulated with PAF, it is possible that overnight fixation could result in an underestimation or failure to detect platelet activation in samples with only minor increases in activation.

Fixation of platelets resulted in increased fluorescence in annexin V samples at all 3 concentrations of formaldehyde in unactivated and activated platelets, compared with results for unfixed samples. In preliminary studies, we found that fixation of platelet samples prior to addition of annexin V resulted in maximal fluorescence that prevented differentiation between unactivated and activated platelet samples (data not shown). Therefore we chose to only evaluate samples fixed after the addition of annexin V. Dörmann et al[14] hypothesized that prior fixation could result in irreversible inhibition of aminophospholipid translocase. Because aminophospholipid translocase is thought to translocate aminophospholipids back from the outer to the inner leaflet of platelet membranes,[17] its inhibition could lead to aminophospholipids remaining in the outer leaflet of platelet membranes and permanent binding of annexin V.[14] It is interesting that the fluorescence associated with incubation with annexin V was not maximal after the addition of formaldehyde. It is possible that formaldehyde caused denaturing of annexin V and limited additional binding. On the basis of the results of our study, we recommend that assessment of equine platelet activation using annexin V should only be performed by use of fresh platelet samples. Fixation should not be used at all.

[a]Sodium citrate, JT Baker Inc, Phillipsburg, NJ.
[b]Fibrinogen polyclonal antibody, Cappel Research Products, ICN Biomedicals Inc, Aurora, Ohio.
[c]IgG $F(Ab^-)_2$ polyclonal antibody, ICN Biomedicals Inc, Aurora, Ohio.
[d]Annexin binding buffer, BD PharMingen, San Diego, Calif.
[e]Annexin V-FITC, BD PharMingen, San Diego, Calif.
[f]ADP Grade I, Chrono-log Corp, Havertown, Pa.
[g]PAF, Sigma Chemical Co, St Louis, Mo.
[h]A23187, Sigma Chemical Co, St Louis, Mo.
[i]FACScan, Becton-Dickinson, San Jose, Calif.
[j]NCSS 2000, NCSS software, Kaysville, Utah.

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