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Original Research

Validation of PAMFix, A Novel Platelet Stabilization Product, for Use on Flow Cytometric Analysis of Pigtailed Macaque (*Macaca nemestrina*) Blood

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Quantification of platelet activation can be important for patients suffering from prothrombotic states, bleeding diatheses, cardiovascular disease, and other diseases in which platelets play a role. The analysis of platelet activation *ex vivo* typically requires blood processing immediately after venipuncture; this requirement can create problematic situations for both medical and research personnel. Flow cytometry is one method used to quantify platelet activation by measuring the expression of platelet surface markers with fluorescent antibodies. PAMFix is a fixative that stabilizes platelet activation markers, including P-selectin (CD62P), in whole blood. PAMFix has already been validated for use in humans and canines for stabilization of whole blood, thus allowing flow cytometry to be performed up to 28 and 22 d, respectively, after venipuncture and reducing the need for expensive equipment and highly trained personnel at the location of venipuncture. Pigtailed macaques (*Macaca nemestrina*) are frequently used in infectious disease research that may require containment conditions that preclude immediate processing of samples. In this study, we tested the efficacy of PAMFix on whole blood from pigtailed macaques to determine the short- and long-term effects of PAMFix on platelet P-selectin expression as analyzed by flow cytometry.

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Introduction

Platelets can be difficult to assess in both research and clinical settings due to their low threshold for activation by different stimuli both *in vivo* and *ex vivo*.[2,9] PAMFix (Platelet Solutions, Nottingham, UK) is a fixative that can be added to whole blood to stabilize platelet activation markers (PAMs), including P-selectin (CD62P), and to prevent further activation of the platelets in the blood during storage.[7] This allows storage and transportation of fixed blood for assessment by flow cytometry at a later date and separate location. PAMFix was originally validated and labeled for use with human blood platelets; studies with platelets activated after venipuncture to test their reactivity and then fixed with PAMFix have shown stable levels of P-selectin on the surface of platelets in human blood for up to 28 d and canine blood for up to 22 d after stabilization.[4,7] PAMFix has also been used in one clinical case of platelet dysfunction in a rhesus macaque.[6] The ability to use PAMFix to ensure sample stability for several weeks after venipuncture is critical when timing does not allow for processing and analysis of blood via flow cytometry within the same day.

Many signaling molecules in platelets are stored in granules and extruded upon receiving activating stimuli.[12]. Platelets are important in the onset and progression of many diseases, including carotid artery atherosclerosis and acute myocardial infarction; therefore, reducing *ex vivo* activation of platelets after venipuncture is critical.[9,10] In addition, platelet activation assays done *ex vivo* are critical tests of physiologic reactivity.[3-5,16] There is a clear need for better platelet fixation methods that allow for flexibility in the data processing timeline, as compared with traditional methodology where the processed sample is evaluated as soon as possible after blood collection.[12]

The technique of flow cytometry poses its own specific challenges. Flow cytometry is the practice of analyzing individual cells for expression of surface markers that are labeled with fluorescently-tagged antibodies using a system of laser excitation and scatter analysis.[8,9,12] Cytometers are expensive to purchase and maintain and can be difficult to use, requiring highly trained individuals for both processing of samples and analysis of results.[12,13] Special care must be taken in protocol design when multiple colors and markers are used in a single protocol to decrease spillover between channels and background staining issues.[1,8,13] Furthermore, there are significant differences among cytometers including excitation sources and detectors, meaning that different fluorescently-tagged antibodies may be required for use with different machines.[13] For studies that involve multiple locations, protocol alterations can mask real and significant changes seen among subjects.

PAMfix has the potential to allow for greater use of flow cytometry in clinical medicine where a flow cytometer is often not available, and similarly allows better control in studies in which patients or animal subjects are sampled at multiple locations. Our goal in this study was to determine whether

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PAMFix adequately stabilizes P-selectin expression on activated and resting platelets in whole blood from pigtailed macaques (*Macaca nemestrina*).

Materials and Methods

On the day before the procedure, flow cytometry tubes (5-mL Falcon Round-Bottom Polystyrene Tubes, Fisher Scientific) were labeled for each animal and appropriately filled with fluorescently-conjugated antibodies: 10 µL of CD42a FITC (BD monoclonal mouse antihuman, clone ALMA.16) and 5 µL of P-selectin (CD62P) PE (BD monoclonal mouse antihuman, clone AC1.2); these antibodies had previously been validated for their ability to recognize these markers on platelets in pigtailed macaque whole blood.[11] CD42a is a membrane marker used to identify platelets. On the day of the procedure, the prepared tubes were moved to room temperature in a biosafety cabinet with the lights off. PAMFix, 2% neutral buffered formalin (NBF), and collagen (Rat tail type 1 collagen, Invitrogen) were put on ice.

Blood was collected from three 7-y-old male pigtailed macaques (*Macaca nemestrina;* animal IDs 337, 338, and 339) to provide 3 biologic replicates. Macaques were sedated with a single injection of ketamine (10 mg/kg IM, Vedco), and 3 mL of whole blood was collected via the femoral vein using a 1.5 inch 21-gauge needle that was inserted directly into a 0.9% sodium citrate vacuum phlebotomy tube (Becton Dickinson). The first vacuum phlebotomy tube of blood was discarded to exclude from analysis any platelets that were activated by the initial vascular puncture. Blood was processed immediately thereafter as described below.

Macaques were single housed, fed a commercial macaque diet (Harlan, Indianapolis, IN), given water ad libitum, and provided with environmental enrichment daily. All procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committee and conducted in accordance with the Animal Welfare Regulations (USDA) and the Guide for the Care and Use of Laboratory Animals (National Academies of Science).

The flow cytometer (FACs Calibur, Becton Dickson, Franklin Lakes, NJ) was calibrated according to standard protocols; 10,000 platelets were collected from each blood sample.

Condition 1–Stabilized blood compared with unstabilized blood. Whole blood was processed for same-day flow cytometry with 4 conditions: with or without PAMFix, and with or without platelet stimulation by collagen (collagen type I from rat tail, Invitrogen), as shown in Figure 1.

For blood without platelet stimulation, 0.5 mL of whole blood was placed in a sterile 1.5-mL screw-cap tube (Sarstedt) containing 1.0 mL of cold PAMFix. The contents were mixed by inversion of the tube, and the tube was incubated for 15 min at room temperature. 150 µL of blood was then added to each designated flow cytometry tube. Whole blood that was not fixed with PAMFix was incubated for 15 min at room temperature in the vacuum phlebotomy tube and then 150 µL was added to each designated flow cytometry tube.

For blood with platelet stimulation, 0.5 mL of whole blood was placed in a 1.5-mL screw-cap tube and 1 uL (final concentration 0.006 mg/mL) of collagen was added. The blood was then incubated for 15 min at room temperature prior to adding 50 µL to each designated flow cytometry tube. Next, 0.6 mL of PAMFix was added to the remaining 0.3 mL of stimulated blood, incubated for 15 min at room temperature, and then 150 µL was added to each designated flow cytometry tube.

Once blood (with or without PAMFix) was added to each flow cytometry tube, it was incubated for 15 min at room temperature, and then 1.0 mL of 2% NBF was added. Blood was incubated with NBF for 30 min at room temperature, and flow cytometry was run on the same day.

Condition 2–Stabilization over time. To test the efficacy of PAMFix at maintaining levels of activation markers on platelets over time, blood was processed as in Condition 1 and placed on a rocker at the lowest speed setting at room temperature prior to flow cytometry on days 9 and 14 after collection. Briefly, 2 mL of whole blood with or without platelet stimulation by collagen (at the same concentration as above) was added to 4 mL of PAMFix in a 15 mL conical (LabForce centrifuge tube, Thomas Scientific) and maintained at room temperature on a rocker. On day 9 and day 14, blood was stained as above and flow cytometry was performed.

Data analysis. After all data were collected using FlowJo (FlowJo LLC, Ashland, OR), a single observer (AJF) who was blind to the sample treatment analyzed the flow cytometry data.

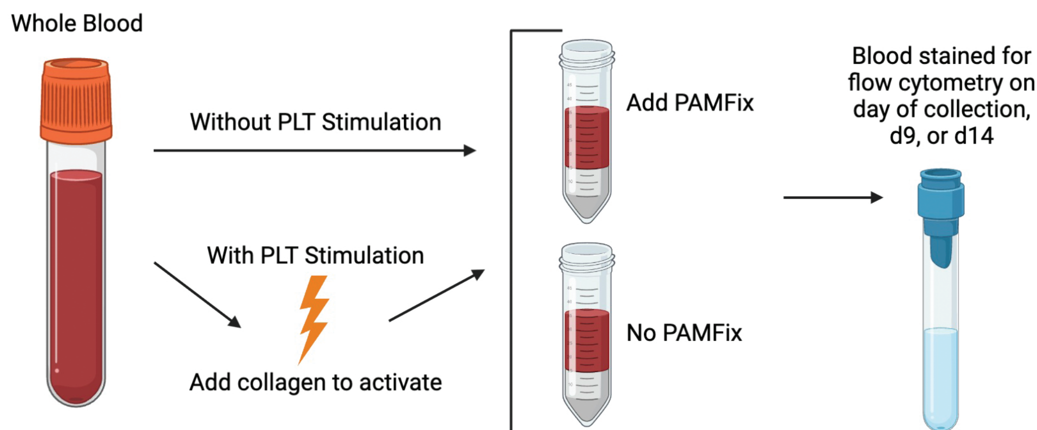

Figure 1. Graphical abstract of blood collection and processing. Unstimulated whole blood was either fixed with PAMFix (cold, 1 volume blood:2 volume PAMFix) or incubated at room temperature without fixation for 15 min, and then stained for flow cytometry. A separate aliquot of whole blood was stimulated with collagen (rat tail type 1, 0.006 mg/mL, 15 min) and then fixed with PAMFix or incubated at room temperature without fixation for 15 min prior to staining. Flow cytometry staining and recording was done on the day of blood collection (d0), and at days 9 and 14 after collection, with room temperature storage of fixed and unfixed whole blood until staining and recording.

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Statistical analysis was performed in GraphPad Prism (GraphPad Software, San Diego, CA) with a significance level of $p<0.05$. Paired *t* tests were used to compare blood P-selectin levels on the same day (day 0) with and without addition of PAMFix. Šidák's multiple comparisons test (mixed-effects analysis) was performed to compare blood P-selectin levels over time with and without addition of PAMFix.

Results

P-selectin levels on pigtailed macaque platelets are unchanged by the addition of PAMFix. We wanted to determine if the addition of PAMFix to unactivated whole blood would change the expression of P-selectin on the surface of platelets. Blood was stained for flow cytometry on the day of venipuncture. We saw no difference in the level of P-selectin on the surface of resting platelets between the PAMFix-treated and untreated samples ($P = 0.73$, Figure 2). We then activated a subset of the remaining whole blood with collagen and tested whether the addition of PAMFix changed the levels of P-selectin between PAMFix and control samples. As before, there was no significant difference ($P = 0.61$, Figure 2).

P-selectin levels on pigtailed macaque platelets are stabilized for up to 14 d by the addition of PAMFix. Previous studies evaluated human or canine whole blood that was activated *ex vivo* prior to PAMFix stabilization and determined that the level of P-selectin expression on platelets was stable for nearly a month after addition of PAMFix.[4,7] We evaluated macaque whole blood that was maintained at room temperature for 9 or 14 days under control conditions or after treatment with PAMFix. Samples were then stained for flow cytometry. Samples treated with PAMFix showed a modest but significant increase in P-selectin expression on day 9 as compared with day 0 (day 0, range 0.1 to 0.4%; day 9, range 1.5 to 1.7%; $P = 0.01$); values on day 14 were not significantly different from day 0 values (range, 1.3 to 2.6%; $P = 0.14$; Figure 3). These ranges represent the percent of platelets (CD42a-positive cells) that were expressing P-selectin (CD62p) in the 3 samples.

Unstabilized blood (stored without PAMFix) showed a statistically significant increase in P-selectin on day 9 compared to day 0 (day 0 range, 0.3 to 0.4%; day 9 range, 3.8 to 5.8%; $P = 0.04$); day 14 values were not significantly different from those measured on day 0 (range 10.1 to 22.9%; $P = 0.39$; Figure 3).

P-selectin expression in whole blood that had been activated with collagen and stabilized with PAMFix showed no significant differences across the test days (day 0 range, 44.6 to 68.7%; day 9 range, 38.4 to 58.1%; $P = 0.22$, and day 14 range, 47.3 to 65.1%; $P = 0.99$ [Figure 3]). Blood that had been activated with collagen and stored without the addition of PAMFix could not be assayed because all samples had clotted by day 9.

Discussion

A growing body of evidence suggests that platelets play a key role in several diseases ranging from mild to life-threatening, and the accurate characterization of the nature of platelet activation requires the ability to measure the level of expression of platelet activation markers at the time of venipuncture.[10,12,17] Collecting accurate data on platelet activation can pose significant challenges where facilities lack appropriate diagnostic tools or expert research staff. Platelets are known to activate *ex vivo* with minimal stimulation, which has traditionally made sending out tests for functional assessment impractical.[17]

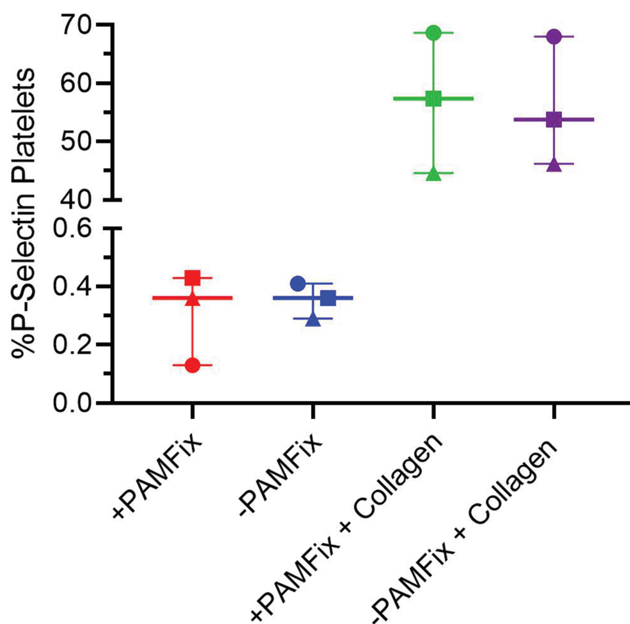

Figure 2. Platelet P-selectin expression on the day of venipuncture (day 0) was not changed by addition of PAMFix. Unstimulated blood (at left, in red and blue) had expression levels between 0.13% and 0.41% ($P = 0.73$). Collagen stimulated blood (at right, in green and purple) had expression levels between 44.6% and 68.7% ($P = 0.61$). Data points represent samples taken from individual animals (different symbol for each animal) and are shown with the 95% confidence interval.

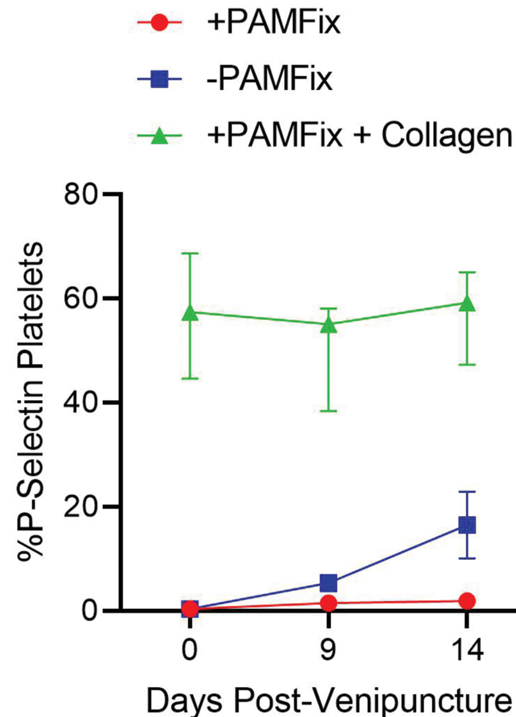

Figure 3. Platelet P-selectin expression was stabilized by PAMFix over time. Unstimulated, unfixed blood (blue) had a substantial increase in expression levels over time (13-fold average increase between baseline and day 9; 44-fold average increase between baseline and day 14) while unstimulated, fixed blood (red) had a negligible increase (4- fold increase between baseline and day 9; 5-fold increase between baseline and day 14). Collagen stimulated, fixed blood (green) had consistent expression levels over time (no increase between baseline and days 9 or 14). Collagen stimulated, unfixed blood clotted in the vacuum phlebotomy tube prior to day 9 and therefore could not be analyzed after day 0. Data are represented as median and error with 95% confidence interval.

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PAMFix is a fixative that has previously been shown to stabilize P-selectin (CD62P) expression on the surface of human and dog platelets for a significant period of time after venipuncture, allowing samples to be transported at room temperature to a facility with a flow cytometer and trained staff for assessment. To test the efficacy of PAMFix in stabilizing surface expression of P-selectin on blood from pigtailed macaques for a period of 14 d, we compared P-selectin expression on fresh activated and unstimulated platelets to P-selectin expression on activated and unstimulated platelets that had undergone fixation with PAMFix. The addition of PAMFix to whole blood did not alter the surface expression of P-selectin in either resting or collagen-activated platelets and was more effective in stabilizing P-selectin expression on resting platelets as compared with sodium citrate anticoagulant alone. Thus, consistent with prior reports in human and canine blood, PAMFix may reduce the risk of misdiagnosis of platelet activation that occurs due to *ex vivo* platelet activation in macaque blood samples.

To determine if PAMFix was effective in stabilizing P-selectin expression on activated and resting platelets for 9 or more days, whole blood was either fixed with PAMFix or left in sodium citrate anticoagulant, stored at room temperature, and tested on days 9 and 14 after venipuncture to track changes in P-selectin expression over time. The unstimulated PAMFixed blood showed a slight increase in expression of P-selectin on days 9 and 14, but the amount of expression did not exceed 2.6% in any sample and remained consistent with normal, unactivated whole blood P-selectin expression from pigtailed macaques based on previously published data.[11] When unstimulated blood was not fixed with PAMFix, we observed an increase in expression on day 9 (3.75 to 5.83%) and a further increase on day 14 (10.1 to 22.9%); these values are high enough to be interpreted as platelet activation which could lead to misdiagnosis or a need for repeat testing. This may not be practical for some studies that use macaques, as venipuncture of macaques and other translational animal models in research facilities often requires chemical sedation and is limited to a certain number of sampling days over a given time period.

It remains to be determined if PAMFix can be effective in stabilizing the surface expression of activation markers other than P-selectin on macaque platelets, and if the stabilization of these markers can be sustained for longer than 14 d. PAMFix has been shown to effectively stabilize human blood expression of surface P-selectin for 28 d and CD63, a dense granule component, for at least 3 d. Constituents of platelet α granules (for example, P-selectin and CD40L) as well as PAC-1, a molecule that binds to the activated configuration of the surface integrin GP IIb/IIIa complex, are routinely measured in macaque studies to assess *in vivo* platelet activation.[11] Verifying the efficacy of PAMFix for stabilization of different platelet surface markers of interest for a sustained time in NHPs would broaden analysis windows for research and clinical applications.

Determining whether transportation of PAMFixed samples adversely affects its efficacy has been examined for human and canine blood, and analogous studies with NHP samples could increase confidence in shipping samples between facilities for multicenter research projects or the use of national primate research resource center core facilities. The current study should be reproduced with additional individual macaques at other institutions. Also, validation in other NHP species could allow for this fixative to be used more widely throughout the NHP research community, as other species including rhesus macaques (*Macaca mulatta*) and common marmosets (*Callithrix jacchus*) are used more commonly than pigtailed macaques (*Macaca nemestrina*). Finally, other options that are available for platelet fixation, such as low concentration calcium-free formaldehyde[14] and ThromboFix platelet stabilizer,[15] should be evaluated alongside PAMFix to determine whether some conditions warrant the use of one or another fixation agent for a given platelet marker or disease state.

In conclusion, we showed that PAMFix can stabilize surface expression of P-selectin in pigtailed macaque blood platelets for up to 14 d at room temperature. As we continue to learn more about platelets and their role in both thrombosis and immune regulation, having technology that allows more flexible analysis timelines is critical for research and clinical case management.

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